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AQUATIC RESEARCH

Vol. 3 Issue 1 Page 1-65 (2020)

Contents/İçerik

Evaluation of Spirulina (*Spirulina platensis*) wastes and live housefly (*Musca domestica*) larvae as dietary protein sources in diets of Oreochromis niloticus (Linnaeus 1758) fingerlings/ 24-35

Cayen Sédro Alofa, Alphonse Adite, Youssouf Abou

AQUATIC RESEARCH
AQUATIC RESEARCH
E-ISSN 2618-6365

Aquatic Research 3(1), 1-12 (2020) • https://doi.org/10.3153/AR20001 Research Article

Evaluation of sucrose as carbon source in mixotrophic culture of *Arthrospira platensis* Gomont 1892

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ABSTRACT

Cyanobacteria are photosynthetic microorganisms that use CO_2 as carbon source and sunlight as energy source. Although phototrophic cultivation is widely used in cyanobacterium production, heterotrophic and mixotrophic cultivations attract attention among researchers. In this study the effect of different concentrations (0[control] - 0.25 - 2.5 - 10 - 50 mM) of sucrose on the growth of *Arthrospira platensis* under mixotrophic cultivation was investigated. The purpose of this study was to investigate whether *A. platensis* biomass production could be performed regardless of high light intensity. Biomass, chlorophyll, lipid and carbohydrate contents were determined by spectrophotometrically. Also the physicochemical properties of the produced cyanobacterium were investigated by FTIR, TGA and DSC. The highest biomass productivity was detected as 1.33 g/L/day in the medium containing 2.5 mM sucrose and the specific growth rate increased 1.32 fold as compared to phototrophic culture. Additionally, the highest lipid content (3.68 ±0.17 mg/g cell) was determined in the same medium. This suggests that *A. platensis* has adapted to the medium that contains low sucrose concentrations. Also, this study showed that sucrose containing medium supports lipid production.

Keywords: Sucrose, Mixotrophic culture, Phototrophic culture, *Arthrospira platensis,* Lipid production

Introduction

Cyanobacteria are photosynthetic microorganisms that use CO_2 as a carbon source and sunlight as energy (Katiyar et al., 2017; Patel et al., 2017). In laboratory scale this natural production type is called phototrophic culture. Although phototrophic cultivation does not need organic carbon source, this culture type makes slow cell growth, low biomass, and higher harvesting cost (Gim et al., 2016; Ozturk Urek & Kerimoglu, 2019). While phototrophic cultivation is widely used in the laboratory, pilot and industrial scale, cyanobacterium cultivation is also performed in heterotrophic cultivation, which contains an external carbon source (Joannesa et al., 2016). In heterotrophic cultivation, cyanobacterium cells are grown in the presence of external carbon source (acetate, glucose, sucrose etc.) but no light (Meireles et al., 2017). The medium in which both CO_2 and an external organic carbon source are present as carbon sources are mixotrophic cultivations. Some microalgae, such as *Chlorella regularis*, *Nannochloropsis* sp., *Synechococcus* sp., *Anabaena* sp., *Arthrospira platensis*, can grow better under mixotrophic condition, which may combine the advantages of phototrophic and heterotrophic cultures (Zhan et al., 2017). The advantages of mixotrophic cultivation are higher growth rate, higher biomass and lipid accumulation, sustain of pigmentation and phytochemicals production, decreased production of CO_2 while there are some problems such as higher cost because of organic carbon source, contamination risk, and reduced energy conversion efficiency (Van Wagenen et al., 2015; Wang et al., 2017; Zhan et al., 2017). When compared with heterotrophic culture, the biomass production in mixotrophic cultivation is not only dependent on the carbon source type and amount. Similarly, in mixotrophic cultivation, there is no need for light intensity as high as in phototrophic cultivation, and light dependence is lower (Abreu et al., 2012). As in mixotrophic growth cyanobacteria showed different metabolic activity from phototrophic culture. Photosynthesis and aerobic respiration are stimulated simultaneously in mixotrophic cultures. Mixotrophic growth offers increasing microbial cell concentration in addition to protein, carbohydrate and lipid productivity. Therefore, mixotrophic cultivation is more economical and easier to control than the other two cultivation types.

In a study growth, lipid and biomass productivity of *Chlorella vulgaris* and *Leptolyngbya* sp. in heterotrophic and mixotrophic regimes were investigated (Silaban et al., 2014). Dextrose and sodium acetate were used as external carbon source and the highest biomass productivity (156 g/m^3d) and neutral lipid productivity (24.07 g/m^3d) was detected with 2.1 g/L sodium acetate in mixotrophic culture. In a study of Ceron Garcia et al., (2006) *Phaeodaciylum tricornutum* was grown in mixotrophic culture which contains fructose, glucose, mannose, lactose or glycerol as external carbon source. Glycerol (0.1 M) was detected as the best substrate that increased final biomass level by 7 fold relative to control cultures. *Arthrospira platensis* cyanobacterium and *Chlorella homosphaera* microalgae were cultivated with glucose in mixotrophic conditions and resulting in biomass increases of up to 3.45 and 2.79 fold, respectively (Margarites et al., 2017).

Since the cyanobacteria *Arthrospira* sp. has important nutritional properties with high protein, essential amino acid and vitamin content, it is an important fish diet alternative (Rosas et al., 2018; Sivakumar et al., 2018). *Arthrospira* sp. can utilize organic carbon substrates in heterotrophic and mixotrophic conditions (Marquez et al., 1993). The blue-green algae *A. platensis* grows mainly on inorganic carbon source and much work has not been carried out on the utilization of organic carbon sources. Some of monosaccharides and disaccharides such as glucose, fructose, sucrose and lactose have been used for mixotrophic cultivation of cyanobacterium and different transport and assimilation mechanisms may be effective for each sugar (Chojnacka & Marquez-Rocha, 2004).

A. platensis is suitable as a biotreatment material for fish production effluents which shows adaptation in mixotrophic cultures. In a study, *A. platensis* was inoculated to the fish culture effluent in order to remove the dissolved nutrients (Nogueira et al., 2018). The concentration of ammonia, nitrite, nitrate and phosphate was detected lower by more than 94.8%, and maximum *A. platensis* productivity was determined as 0.03 g/L. day.

In the study of Chojnacka and Noworyta (2004), the influence of growth parameters on specific growth rate of *Arthrospira* sp. in photoautotrophic, heterotrophic and mixotrophic batch modes were investigated and the highest specific growth rate (0.055 h^{-1}) was reached in mixotrophic culture with 2.5 g/L glucose.

Some studies have thus focused on finding cheaper organic carbon sources to decrease production cost (Bhatnagar et al., 2011; Lin & Wu, 2015). Sucrose present in the waste of the sugar production process is an important alternative carbon source (Abreu et al., 2012; Wang et al., 2016). The use of sucrose-containing waste as a carbon source will evaluate of a waste material and provide low cost production (Mitra et al., 2012). Therefore, it is important to investigate the growth and production aspects of cyanobacterium in sucrose containing medium.

In this study *A. platensis* was cultivated under mixotrophic cultivation with different concentrations of sucrose as a carbon source. Effects of carbon source's concentration on production of biomass, chlorophyll, and total lipid were investigated. Also specific growth rates were calculated. The aim of this study was to investigate the effect of sucrose concentration on growth and lipid production of *A. platensis.* The lipid production of *A. platensis* in sucrose-containing growth medium was investigated for the first time in this study. Also the characterization of produced cyanobacterium cell with TGA and FTIR is a novel approach.

Material and Methods

Cyanobacteria and Culture Media

The cyanobacteria *Arthrospira platensis* (Gomont) 1892 was provided from Cukurova University, Faculty of Aquaculture, Adana-Turkey. For the maintenance of cyanobacteria under phototrophic culture, it has been growth in Zarrouk's medium (Zarrouk, 1966). Batch cultivation was carried out in 750 mL medium at 2500 lux (33.75 µmol photon m^{-2} s^{-2}) light intensity (by white fluorescent lamps) with continuous illumination, pH 9.0 and 30°C and the cultures were mixed and aerated using filtered air continuously.

Mixotrophic Cultivation

Mixotrophic culture was carried out in Zarrouk's medium, which contained different concentration of sucrose (0 [control] – 0.25 – 2.5 – 10 – 50 mM) as carbon source. Culture was inoculated to an initial optical density (OD) of 0.2 at 600 nm (Vonshak et al., 1982). OD is a parameter used to determine biomass production. When working with filamentous microorganisms, make sure that the culture medium is well mixed before reading the OD. In this present study, well mixed *A. platensis* culture was transferred to spectrophotometer cuvette and the cuvette was turned upside down for three timed and then OD was read.

Batch cultivation was carried out in 250 mL Erlenmeyer with 100 mL working volume at 1500 lux (20.25 µmol photon m^{-2} s^{-2}) light intensity (by white fluorescent lamps) with continuous illumination, 100 rpm shaking rate (Thermoshake Incubator, Gerhardt, Germany), pH 9.0 and 30°C. OD, pH, chlorophyll and total lipid content were detected during incubation period. Zarrouk's medium without any external carbon sources was used as control condition.

Specific growth rate (µ) and biomass productivity (P) were calculated according to Eq. 1 and 2 based on OD values (Kong et al., 2013) (X: amount of microorganism, t: time as day).

$$\mu = \ln\frac{X1-X0}{t1-t0} \qquad \text{Eq. 1}$$

$$P = \frac{X1-X0}{t1-t0} \qquad \text{Eq. 2}$$

Determination of Chlorophyll Content

Chlorophyll a and b contents were measured as described by Lichtenthaler and Wellburn (1983). 5 mL of algal suspension was centrifuged at 5000 rpm for 15 min. Pellet was weighted and homogenized in 5 mL absolute ethanol by 8000 rpm for 1 min and 9500 rpm for 1 min with 30 seconds intervals with laboratory homogenizer (Ultra Turrax, IKA, Germany). After centrifugation absorbance of the obtained supernatant was measured at 470, 664.2 and 648.6 nm. Chlorophyll a and b contents were calculated according to Eq. 3 and 4 (Lichtenthaler & Wellburn, 1983).

$$\text{Chl a} = 13.36 \times Abs_{664.2} - 5.19 \times Abs_{648.6} \qquad \text{Eq. 3}$$

$$\text{Chl b} = 27.43 \times Abs_{648.6} - 8.12 \times Abs_{664.2} \qquad \text{Eq. 4}$$

Determination of Total Lipid Content

Total lipid content of cyanobacterium was determined by Mishra et al., (2014) method. To prepare reagent 0.6 g vanillin was dissolved in 10 mL ethanol and mixed 90 mL distilled water and 400 mL concentrated phosphoric acid. 2 mL concentrated sulfuric acid was added to 100 µL cyanobacteria sample and was heated for 10 min at 100°C, and was cooled for 5 min in ice bath. 5 mL of freshly prepared phospho-vanillin reagent was then added and the sample was incubated for 15 min at 37°C incubator shaker at 150 rpm. The absorbance was measured at 530 nm against a reference sample.

Determination of Total Carbohydrate Content

Total carbohydrate content of production medium was determined by phenol-sulphuric acid method (Dubois et al., 1956). To determine total carbohydrate content, 1 mL cell free supernatant (1 mL distilled water for reference) was mixed with 1 mL 5% (w/v) phenol solution and 5 mL concentrated H_2SO_4. After well mixing the samples were incubated for 20 min at room temperature the absorbance was measured at 470 nm against a reference sample. Glucose was used as standard in the range of 0- 250 µg/mL.

TGA and FTIR Analysis

TGA and DSC analyses of produced cyanobacterium in phototrophic and mixotrophic cultures were carried out with Perkin Elmer- Diamond TG/DTA (Massachusetts, USA). About 3-5 mg of dry produced cyanobacterium cell sample was loaded on a platinum pan and its energy level was scanned in the ranges of 30 - 500°C under a nitrogen atmosphere with a temperature gradient of 10°C/min.

To analyze the organic structure of produced *A. platensis* cell, the FT-IR spectra were recorded on the Perkin Elmer Spectrum BX (Massachusetts, USA), in the 4000- 400 cm^{-1} spectral region with deuterated triglycine sulfate detector. All samples were dried at 70°C overnight before analysis. KBr pellet was used as a back ground reference. Approximately 1 mg of the sample was milled with approximately 100 mg of dried KBr and then pressed to form a pellet for measurement.

Statistical Analysis

All experiments were carried out in triplicates (n=3) and repeated 3 times. Each value is an average of 3 parallel replicates. Data were presented as mean±standard deviation. The data were analyzed by analysis of variance (ANOVA) to identify the significantly different groups at ($P<0.05$) by one-way ANOVA test using SPSS software statistical program (SPSS for windows ver. 21.00, USA).

Results and Discussion

In this present study *A. platensis* was grown in five different media which contain variable concentration of sucrose as carbon source. OD values were determined depending on sucrose concentration changes (Figure 1). These results suggest that the *A. platensis* is adapting to the mixotrophic condition. At low sucrose concentrations, stationary phase was reached in later days (16^{th}) of incubation. At lower sucrose concentrations of less than 2.5 mM, the specific growth rate was lower, while the rising sucrose concentrations increased the specific growth rate (Figure 2). The highest specific growth rate (0.118 day^{-1}) was detected in 2.5 mM sucrose medium ($p<0.05$). Similarly, in a study the highest specific growth rate was detected in mixotrophic cultivation with 2.5 g/L glucose (Chojnacka, & Noworyta, 2004).

In other production media the specific growth rates were detected as 0.091 day^{-1} (with 0 mM sucrose), 0.102 day^{-1} (with 0.25 mM sucrose), and 0.046 day^{-1} (with 10 mM sucrose), (as the specific growth rate with 50 mM sucrose medium was lower, it was not shown in graph). In medium with high sucrose concentration (10 or 50 mM), the cell has mass growth and may not have gone into cell division. 2.5 fold decrease was detected in specific growth rate with 4 fold increasing sucrose concentration ($p<0.05$). In mixotrophic culture, autotrophic and heterotrophic metabolism were work together. The cyanobacteria cells were reached stationary phase rapidly and there were no significant changes in OD values. For this reason, specific growth rate of the medium with high sucrose concentration was detected lower than control condition (phototrophic cultivation).

In this present work, a higher growth rate was achieved in the mixotrophic medium than in the control condition because of low light intensity (1500 lux) and the presence of external carbon source. Even in phototrophic cultivation of *A. platensis* the optimal light intensity is 2500 lux, the light intensity in control condition (1500 lux or 20.25 µmol photon m^{-2} s^{-2}) was deficient. The light intensity in mixotrophic culture was sufficient as there was external carbon source. At high sucrose concentrations, substrate inhibition was also determined. The growth rate in the mixotrophic medium containing 10 mM sucrose was about 2 times lower than in the control condition ($p<0.05$). As a result of reaching rapidly to the specific growth rate, substrate inhibition was detected.

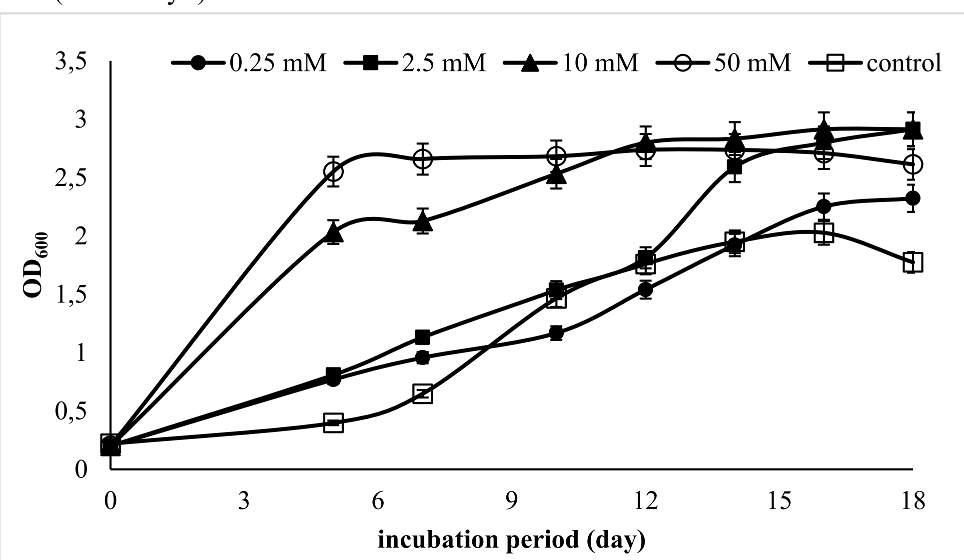

Figure 1. Variations of OD in *A. platensis* in different sucrose concentration medium depending on incubation period.

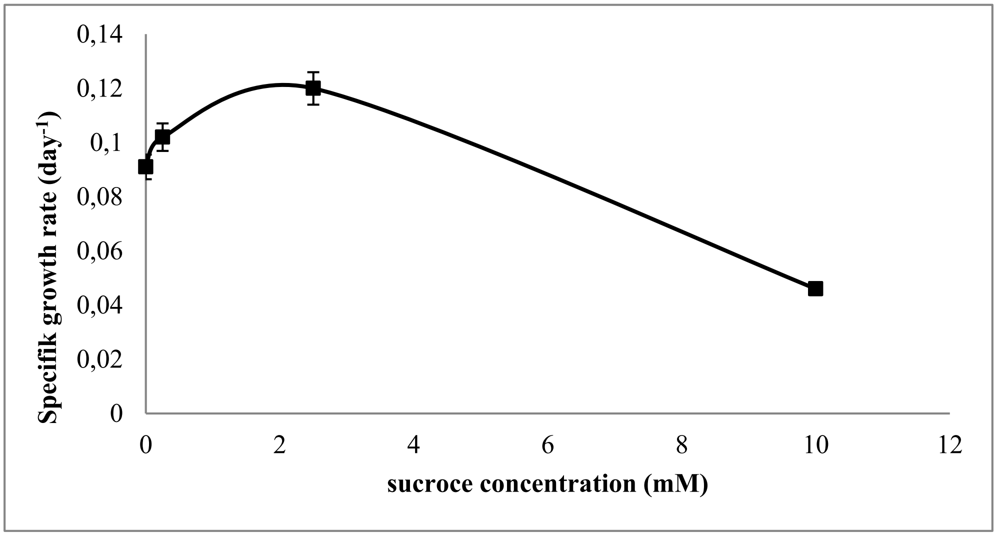

Figure 2. Specific growth rate values in varying sucrose concentrations media.

Additionally, the highest biomass productivity was detected as 1.33 g/L/day in the medium containing 2.5 mM sucrose. The biomass productivity of *Arthrospira* sp. varies from 0.06 to 4.3 g/L/day depending on the species (Mata et al., 2010). This result shows that *A. platensis* adapted to sucrose containing medium (2.5 mM) and reached to a high production rate. An increase was detected in biomass productivity with increasing sucrose concentration up to 2.5 mM. These results indicate that *A. platensis* cannot be adapted to high sucrose concentrations. However, the sucrose concentration up to 2.5 mM provided better growth than the control condition. When the medium supplemented with external carbon source, C availability can exceed cell necessities for growth and the rest carbon is directed towards lipid or carbohydrate synthesis (Lari et al., 2016). Generally specific growth rate of mixotrophic culture is the sum of phototrophic and heterotrophic metabolism because the external organic carbon promotes faster growth (Perez-Garcia et al., 2011). It can be said that, under controlled condition the specific growth rate is lower in the mixotrophic medium containing sucrose up to 2.5 mM than the controlled media ($p<0.05$).

Different kinds of simple sugars like glucose, fructose, galactose, mannose, lactose and sucrose support the mixotrophic and heterotrophic growth of cyanobacteria with species-specific differences in uptake and assimilation mechanisms (Neilson & Lewin, 1974; Shi et al., 1999; Sun et al., 2008). The study of the effect of different sugars and concentrations on the growth of *Arthrospira* sp. have shown that sucrose does not support growth in the dark but is effective in growing for certain species in the light conditions (Mühling et al., 2005). In this study, bleaching was detected in *A. platensis* cultures during adaptation to sucrose medium, and present study shows similarity in high sucrose concentrations (50 mM). Sucrose, trehalose and glucosyglycerol are osmoprotective compounds. The cyanobacterium *Synechocystis* sp. has active transport mechanism for glucosyglycerol and in salt-adapted cells is mainly achieved by *de novo* synthesis of the transport system (Mikkat et al., 1996; Mikkat et al., 1997). The studies support that trehalose and sucrose are taken up by the cells and possesses nearly the same as glucosylglycerol.

The inhibitory effect of the 50 mM sucrose concentration is also evident from the level of Chl-a and Chl-b content (Figure 3-4). The highest Chl-a content (301.173 ±14.8 mg/ g cell) was detected in the 2.5 mM sucrose containing medium, while the highest Chl-b content (42.62 ±1.9 µg/ mg cell) was detected under phototrophic cultivation. The chlorophyll amount did not differ significantly between control condition and mixotrophic cultivation (with 2.5 mM sucrose). In the study of Gim et al. (2016), the chlorophyll concentrations had no meaningful changes in mixotrophic (with 20 mM glucose) and phototrophic cultures.

In phototrophic culture the sole carbon source was CO_2 and the cyanobacteria needed chlorophyll to produce nutrient by using light and CO_2. On the contrary, in mixotrophic condition CO_2 was not the unique factor that supported biomass production. The mixotrophic condition is identified as "two-stage" mode (Zhan et al., 2017). The first stage is heterotrophy due to high content of initial organic carbon. When the organic carbon reduces to a certain level, phototrophic metabolism gets involved as first stage. Increasing amount of Chl-a in the late days of incubation under mixotrophic condition is associated with an increase in the amount of cells and phototrophic metabolism. And also the decreasing of available organic carbon load in the medium turns the mixotrophic metabolism to phototrophic metabolism.

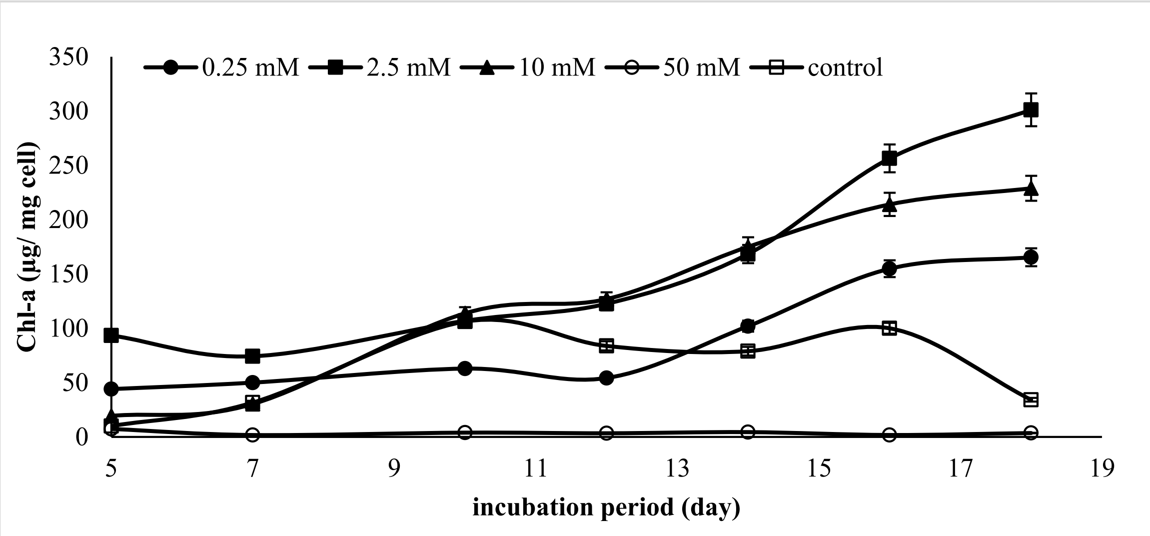

Figure 3. Chlorophyll-a content of *A. platensis* in different sucrose concentration medium depending on incubation period.

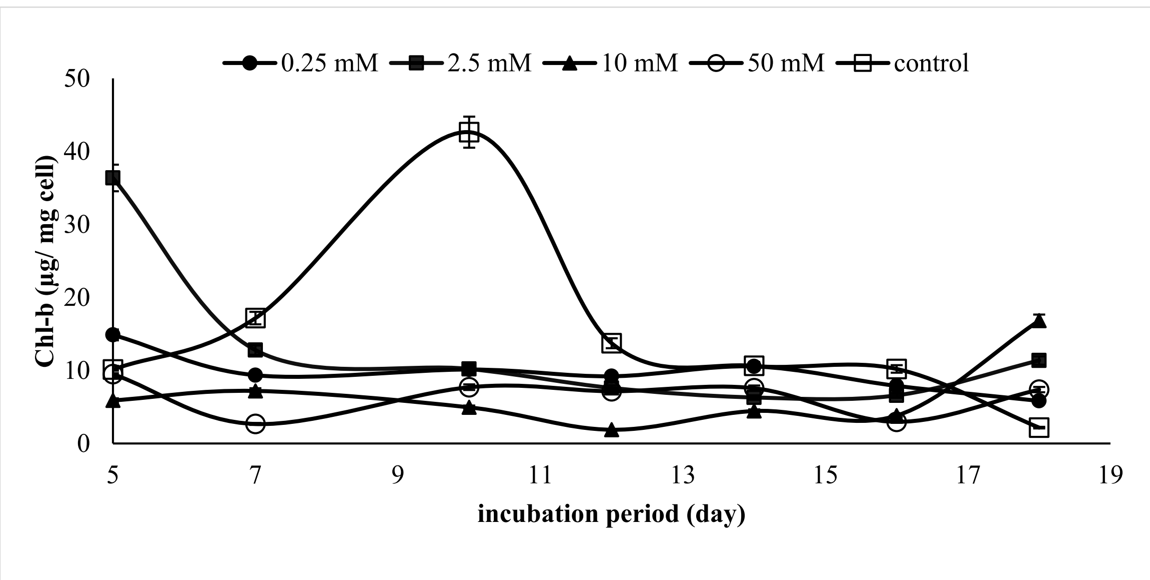

Figure 4. Chlorophyll-b content of *A. platensis* in different sucrose concentration medium depending on incubation period

There was approximately 1.5 unit overall increase in pH values during the incubation period (Figure 5). The determined pH value increase may be due to organic bases released into the medium during the production process. Only in the presence of 50 mM sucrose, there was a decrease with a fluctuation in the pH value. In this medium, excess carbon source may inhibit the cellular metabolism, hence the disaccharide is not rapidly breakdown.

Insignificant lipid production was detected at minimum (control and 0.25 mM) and maximum (50 mM) sucrose concentrations ($p>0.05$) (Figure 6). While lipid production varied with increasing sucrose concentration, the highest lipid content (3.68 ±0.17 mg/ g cell) was determined on the 16th day of incubation in medium containing 2.5 mM sucrose ($p<0.05$). In control condition and the medium containing 0.25 mM sucrose, the maximum amount of lipid was detected in the first days of incubation. In medium with high sucrose concentration, lipid production was increased late in the incubation days because the cells provided later adaptation. When the sucrose concentration was higher than 2.5 mM, substrate inhibition was observed. In a study two different microalgae were grown in mixotrophic culture including glucose (Cheirsilp & Torpee, 2012). The lipid content of both strains decreased sharply when the initial glucose concentration increased from 0 to 4 g/L. At above 4 g/L of initial glucose concentration, the lipid content did not change significantly. Similarly, in the study of Lin and Wu (2015), lipid production of *Chlorella* sp. increased when the initial sucrose concentration increased to 0.5 g/L. When the initial sucrose concentration was higher than 0.5 g/L, the lipid production decreased.

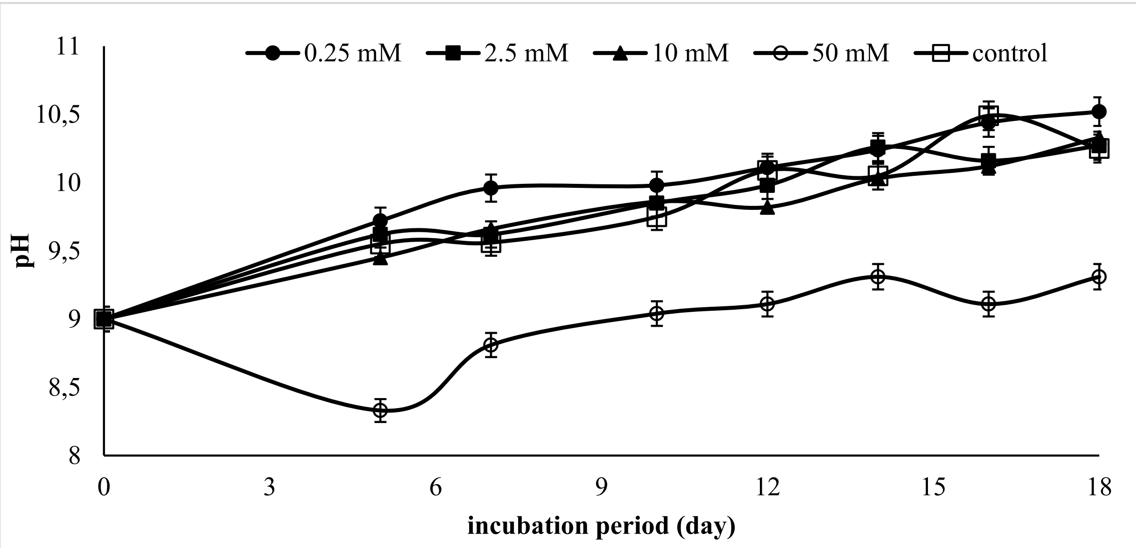

Figure 5. Variations of pH of *A. platensis* in different sucrose concentration medium depending on incubation period.

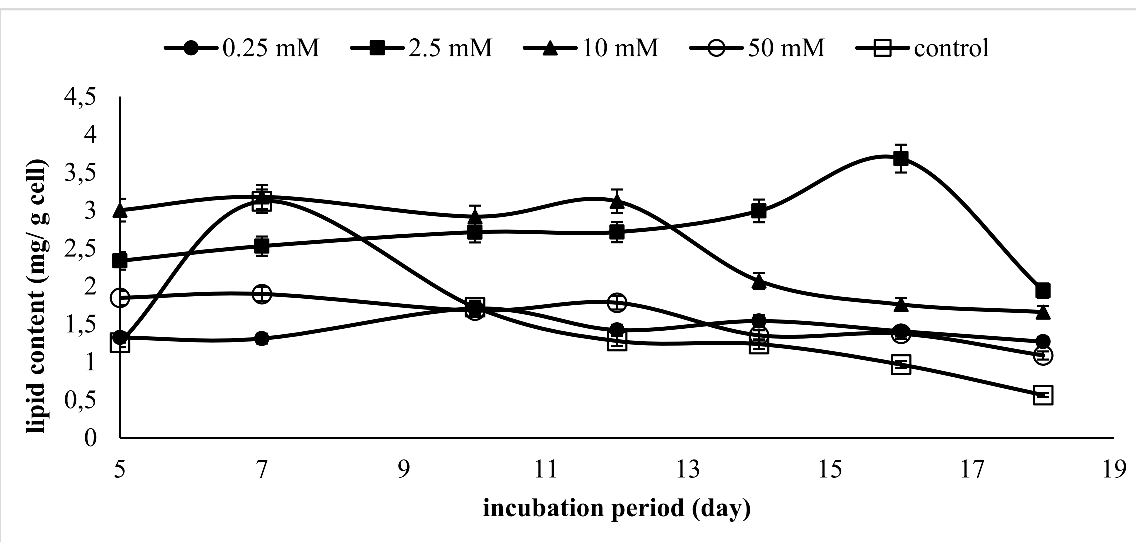

Figure 6. Lipid content of *A. platensis* in different sucrose concentration medium depending on incubation period.

Table 1. Comparison of control condition (phototrophic) and mixotrophic culture (containing 2.5 mM sucrose) that has been determined the best results.

Parameters	mixotrophic culture	control (phototrophic culture)
Specific growth rate (day^{-1})	0.118	0.091
Biomass productivity (g/L/day)	1.33	0.153
Lipid content (mg/ mg cell)	3.68 ±0.17	3.118 ±0.14
Chl-a content (µg/ mg cell)	301.173 ±14.8	106.303 ±4.9
Chl-b content (µg/ mg cell)	36.362 ±1.7	42.62 ±1.9

Values are mean ± S.D., N = 3; ($p < 0.05$).

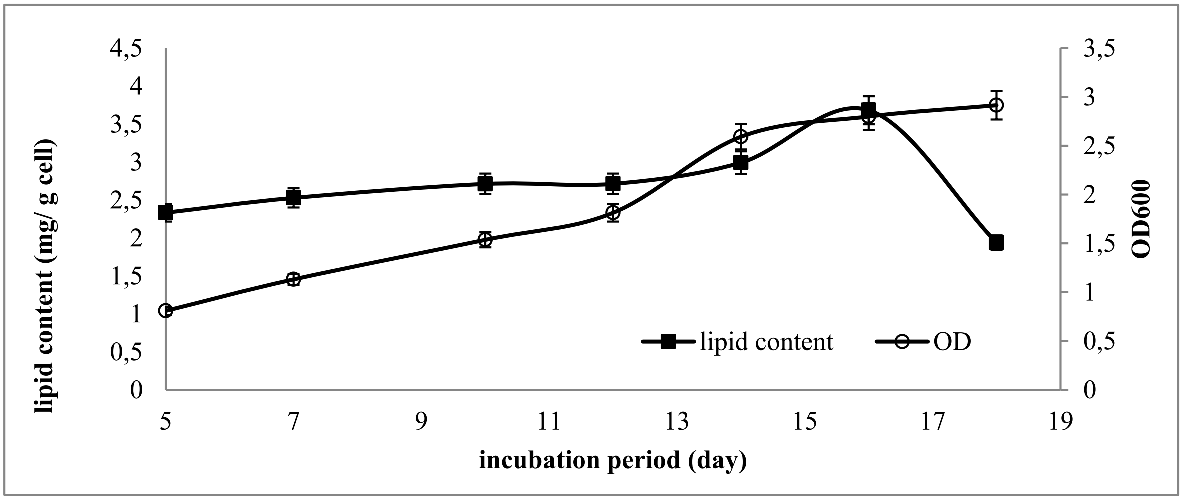

Figure 7. Lipid content and OD of *A. platensis* in 2.5 mM sucrose medium depending on incubation period.

According to the obtained results, the highest specific growth rate, biomass productivity, Chl-a and lipid content was detected in mixotrophic culture that contains 2.5 mM sucrose (Table 1). These results showed that sucrose including medium supports biomass and chlorophyll production and lipid accumulation of *A. platensis*.

In the highest lipid production condition, biomass and lipid content according to the incubation period is shown in Figure 7. According to figure it was determined that the maximum amount of lipid was obtained at the stationary phase. This can be interpreted as *A. platensis* culture grown with adaptation of sucrose has increased lipid production by entering the stress due to reduced external carbon source in the medium. In this medium on the 16^{th} day of incubation, external and intracellular total carbohydrate concentration was detected as 28.29 ppm and 130.26 µg/g cell, respectively.

The thermal stability of the produced cell was investigated by TGA and DSC. The thermal stability of produced cell could show difference according to production medium. The produced cyanobacterium cells in different media have almost same degradation profile. In the first step, 2.19% of weight loss for phototrophic production and 4.13% of weight loss for mixotrophic production were recorded. The maximum degradation was determined in the second step for two of them. The weight loss was 52.94% and 54.74% for phototrophic and mixotrophic production, respectively. The most important difference was the cyanobacterium cell that produced in phototrophic culture has showed more rapid weight loss than produced in mixotrophic culture. That means the cyanobacterium cell produced in mixotrophic culture has higher thermal stability.

Also the functional groups of the produced cyanobacterium were investigated by FTIR. In FT-IR spectra of cyanobacterium cell produced phototrophic and mixotrophic cultures there were same peaks such as 2990-2924 cm^{-1} showed CH_3 asymmetric stretching which was associated with lipid, carbohydrate or protein structure, 1650 cm^{-1} relevant with C=O stretching on protein structure and the peak belongs to N-H bending and C-N stretching next to 1542 cm^{-1}, 1240 cm^{-1} related to asymmetric stretching of hydrocarbon chain and phospholipid structures. The peak at 2856 cm^{-1} was formed as a result of CH_2 symmetric resonance in lipid and carbohydrate structure which was detected only cyanobacterium cell produced in mixotrophic culture FTIR spectrum. This result supports that more lipid production was produced in the mixotrophic culture.

Conclusions

In conclusion the present study suggests a new carbon source for mixotrophic culture of *A. platensis* using sucrose. *A. platensis* has adapted to the medium that contains low sucrose concentrations. Significant decrease was detected in specific growth rate with increasing sucrose concentration ($p<0.05$). In the mixotrophic cultivation of *A. platensis* two different metabolic pathways were active. Due to there was sufficient external carbon source in the first days of incubation, heterotrophic metabolism was used more actively. When the organic carbon source sucrose reached a critical concentration chlorophyll content started to increase. That means heterotrophic and phototrophic metabolism worked correlated. The produced cells in mixotrophic culture (with 2.5 mM sucrose) has higher thermal stability depending on TGA. Additionally, this study showed that sucrose containing medium supports lipid production. And this result is supported by the FTIR spectrum. In this present study, it can be said that *A. platensis* can use sucrose as a carbon source. This result is an indication that various wastes containing sucrose such as molasses, sugar cane bagasse can also be used as a carbon source in the production medium. Thus, valuable products such as biomass, protein, and lipid can be produced more economically and hence used economically for in various industrial areas such as food, fisheries, and pharmaceuticals. The produced *A. platensis* biomass would be evaluated as protein and lipid source in aquaculture diets.

Compliance with Ethical Standard

Conflict of interests: The authors declare that for this article they have no actual, potential or perceived conflict of interests.

Ethics committee approval: There is no need ethics committee approval.

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Removal of high concentration of nitrate and phosphate from aqueous mixotrophic solution by *Chlorella vulgaris*

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ABSTRACT

Microalgae exhibit large potential as an alternative to advanced biological nutrient removal in wastewater or simulated wastewater at laboratory conditions. Therefore, it is necessary to determine the optimum conditions for nutrient removal. This study investigated the total carbohydrate, chlorophyll-a, -b, carotenoid and lipid production and nutrient removal of mixotrophic microalgae (*C. vulgaris*) cultured in different nitrate/phosphate rich modified BG-11 medium (0-200 mg L^{-1}) at longer growth periods (10 days). The mean removal efficiency of NO_3-N (in nitrate source), and PO_4-P (in phosphate source) (88.29 ±0.12 and 31.06 ±0.22%, respectively) was reached in the mixotrophic culture. Under the optimum conditions (200 µmol photon $m^{-2}s^{-1}$ 16 h photoperiod and 28% inoculum size), 63.61-99.05% of NO_3^- and 13.97-63.77% of PO_4^{3-}were successfully removed. The lipid and carbohydrate productivities were 27.95 and 29.53 g $L^{-1}d^{-1}$, 0.2869 and 0.2435 g L^{-1} d^{-1} respectively, which were approximately 9-12 times higher than those in photoautotrophic condition. The BG-11 growth media containing 10 g L^{-1} glucose and excessive amount of nutrient effect results indicate that the Chl-a, -b and carotenoid contents of *C. vulgaris* is higher at 100 mg L^{-1} N and 50 mg L^{-1} P growth media composition compared to 100% growth media composition. Thereby, the findings of this study provided an insight into the role of algal uptake of nutrients under the nutrient rich mixotrophic medium for the future algae-based treatment application.

Keywords: Bioremediation, *Chlorella*, Mixotrophic solution, Nutrient removal, Algal removal

Introduction

The application of microalgae for wastewater treatment has gained much attention due to the potential of microalgae to simultaneously remove nutrients and produce valuable biomass. Their great potential in producing biodiesel, which is a renewable energy source, can reduce the greenhouse gas emissions (Abe et al., 2008; Khan and Yoshida, 2008; Bruce, 2008; Groom et al., 2008; Azianabiha et al., 2019).

The production of biofuels from microalgae is associated with high demands of nutrients required for growth (Barbera et al., 2016). Their lipid productivity/biomass (dry weight) is about 15–300 times that of conventional crops (Chisti, 2008). Therefore, microalgae are considered as a promising substitute for fossil fuels in the future (Li et al., 2010).

Phosphorus is one of the most important nutrient in domestic waste-water. It is difficult to remove and hence along with nitrogen is responsible for eutrophication of water bodies, especially where untreated sewage is discharged. Nutrient removal is becoming a regular approach for wastewater treatment plant, since excess nitrogen and phosphorus in discharged wastewater can lead to downstream eutrophication and ecosystem damage (Swati et al., 2017).

Based on these considerations, it is clear that the only way to obtain an economically and environmentally sustainable microalgal biofuels production is to recycle the nutrients, the majority of which is not included in the lipid fraction destined to biofuels,and remains in the residuals. This possibility is clearly highly connected with the method employed for biomass treatment after harvesting (Sialve et al., 2009; Heilmann et al., 2011; Biller et al., 2012; Rösch et al., 2012; Garcia Alba et al., 2013; Levine et al., 2013; López Barreiro et al., 2013; Zhang et al., 2014; Ward et al., 2014; Barbera, 2016).

Microalgae growth is possible under heterotrophic or mixotrophic conditions as well as autotrophic conditions depending on specific characteristics of the species (Andrade and Costa, 2007) and some microalgae species like *Chlorella vulgaris* (Mitra et al., 2012), *Haematococcus pluvialis* (Kobayashi et al., 1992), *Spirulina platensis* (Marquez et al., 1993), *C. sorokiniana* (Wang et al., 2012), *Botryococcus braunii* (Zhang et al., 2011), and *C. zofingiensis* (Liu et al., 2011) have been observed under autotrophy, heterotrophy, and mixotrophy conditions. Mixotrophic cultivation of microalgae provides higher biomass and lipid productivities than cultivation under photoautotrophic conditions, the cost of the organic carbon substrate is estimated to be about 80% of the total cost of the cultivation medium (Bhatnagar et al., 2011).

The objective of this study was to quantify some biochemical changes (lipids, chlorophyll-a and -b, carotenoids and total carbohydrate and removal of nutrients) in mixotrophic condition (glucose substrate) of *Chlorella vulgaris* grown in nitrate-phosphate rich conditions. Nitrate and phosphate concentrations were measured on the initial and final days of cultivation to evaluate nutrient removal rates. Therefore, the aim of the present study was to determine nutrient uptake performance and efficiency of *Chlorella* cells under the nutrient rich mixotrophic medium for the future algae-based wastewater treatment application.

Material and Methods

Algal Growth Medium and Experimental Design

C. vulgaris was obtained from the Culture Collection of Microalgae at the University of Ege, Izmir, Turkey. The modified and non-modified BG-11 medium were used as the growth medium in the experiments. The growth and nutrient uptake experiments were conducted at four different nutrient levels as presented in Table 1. NO_3-N ($NaNO_3$) and PO_4-P (K_2HPO_4) were used as the nitrogen and phosphorus sources, respectively. A standard initial inoculum of the algae was inoculated to culture flasks (200 mL each) that contained BG-11 medium and incubated at 28 ± 1ºC under 14 h light (20 E m^{-2} s^{-1} ±20 %), with magnetic stirring (100 rpm). For mixotrophic cultures, glucose was added to the culture broth in concentration of 10 g L^{-1} maintaining the same L/D photoperiod of 14:10 h. BG11 medium and BG11 medium containing glucose were used for autotrophic culture and mixotrophic culture of *Chlorella* cells, respectively. 10 g L^{-1} glucose has been proved to be an ideal organic matter source for the mixotrophic cultivation of microalgae in some previous studies (Liang et al., 2009; Cheirsilp & Torpee, 2012). To examine the removal effect of nitrogen and phosphorus from modified medium by using *C. vulgaris* cells, the selected microalgae were triplicate cultured in medium with 0, 50, 100, 200 mg L^{-1} concentration of nitrate and phosphate for 10 days. The initial pH was adjusted to 7 using 10% HCl and the contents of chlorophyll-a, chlorophyll-b, lipid and carotenoids in the supernatant were determined by UV-VIS spectroscopy.

Table 1. Initial nutrient levels for batch experiments with *C. vulgaris.*

Experiment	NO_3-N (mg/L)	PO_4-P (mg/L)	Glucose (g/L)
Control* (n-mm Bg-11)	0.06 ±0.003	0.001 ±0.001	No glucose
mm BG-11	0	0	10 ±0.01
	50 ±0.24	50 ±0.77	10 ±0.04
	100 ±0.58	100 ±0.96	10 ±0.05
	200 ±1.33	200 ±1.46	10 ±0.01

*Key to subscripts: n-mm: non modified medium, mm: modified medium.

Determination of Chlorophyll and Total Carotenoids Concentration

Chlorophylls and carotenoids in *C. vulgaris* were extracted with methanol and spectrophotometrically determined as described by Dere et al. (1998). Total pigment content was obtained by summing chlorophylls and carotenoids contents

Lipid Analysis

Lipid contents of the microalgae were directly measured by sulpho-phospho-vanillin (SPV) colorimetric method (Mishra et al., 2014). At the end of the cultivation, algal biomass was harvested to measure lipid content. The relationship between the lipid content of the 100 μL microalgae suspensions and the absorbency at 530 nm was acquired from a previous study (Tao et al., 2017; Eq. 1):

Lipid (mg) = 0.123 X OD_{530} + 0.003 (R^2= 0.999) (1)

Dry Weight and Nutrient Removal Analysis

The dry weight of algal biomass was determined using the method of suspended solid (SS) measurement. For the measurement of water quality, the algal culture was centrifuged (10.000 rpm X 10 min at 4°C) and filtered through a 0.45 µm filter. After that, the weight of *C. vulgaris* was calculated from the calibration curve that obtained from the dry cell weight method (Eaton, 2005). The filtered supernatant was then used for the determination of nitrate and phosphate concentrations. To determine nutrient removal rates, NH_3^+-N and PO_4^{3-} -P were measured on initial and final days of the experimental period. The samples were filtered with a 0.2-µm pore-size membrane filter prior to the measurement to exclude suspended materials. Nutrient removal rate (R, %; Eq. 2) and removal capacities (q, mg/L day, Eq. 3) were calculated as (Babaei et al., 2013):

$R=100 \times (C_i - C_f) / C_i$, (2)

q (mg/L day) = $(C_i - C_f) \times V/m$ (3)

V: Solution volume (mL)

m: Dry weight of the adsorbent (g)

C_i and C_f: initial and final nutrient concentrations of NH_3^+-N or PO_4^{3-}-P on initial and final days of the experimental period, respectively.

All experiments were performed in 3 replicates. The data are presented as the mean±standard deviation of the mean (SDM).

Results and Discussion

Chlorophyll-a and b and Carotenoid Contents

In this work, the effects of mixotrophic medium, which is contain high concentration of nitrate and phosphate, were systematically investigated on *C. vulgaris*, regarding the nutrient uptake, the lipid productivity, the chlorophyll, carotenoid and carbohydrate content.

Chl-a and b and carotenoid levels for the control group were measured 0.6565, 0.9883 and 0.0985 µg/L, respectively under mixotrophic cultivation. At the end of the experiment, the highest chlorophyll-a and -b and carotenoid contents were observed in the 50 mg L^{-1} (1.33 µg L^{-1}) and 50 mg L^{-1} (2.24 µg L^{-1}) and 100 mg L^{-1} (3.57 µg L^{-1}), respectively. Measurements for the Chl-a and b and carotenoid content, for the 100 mg L^{-1} and 50 mg L^{-1} concentration of nitrate and phosphate solution, showed that the high concentration of NO_3^- and PO_4^{3-} treatment causing an increase in Chl-a and b and carotenoid, respectively (Figure 1). Chlorophyll content results showed that 100 mg L^{-1} nitrate treatment caused an increase in Chl-a and b and carotenoid levels, while 50 mg L^{-1} phosphate treatment decreased.

Chlorophyll is one of the cellular compounds on the basis of which microalgal biomass in the culture is estimated and it can be used to measure cell growth (Kong et al., 2013). According to a previous report, the utilization of an external organic carbon source may affect the photoautotrophic growth processes, such as photosynthesis and respiration (Kong et al., 2013). As shown in Figure 1, the effect of glucose and 100 mg L^{-1} and 50 mg L^{-1} concentration of nitrate and phosphate solution on the photosynthetic pigment content and productivity of mixotrophic *C. vulgaris* was significant. Our results showed that the mixotrophic cultures experience an increase in photosynthetic pigment productivity that was dependent on the increase of high concentration of nutrient in the medium content (Kong et al., 2013).

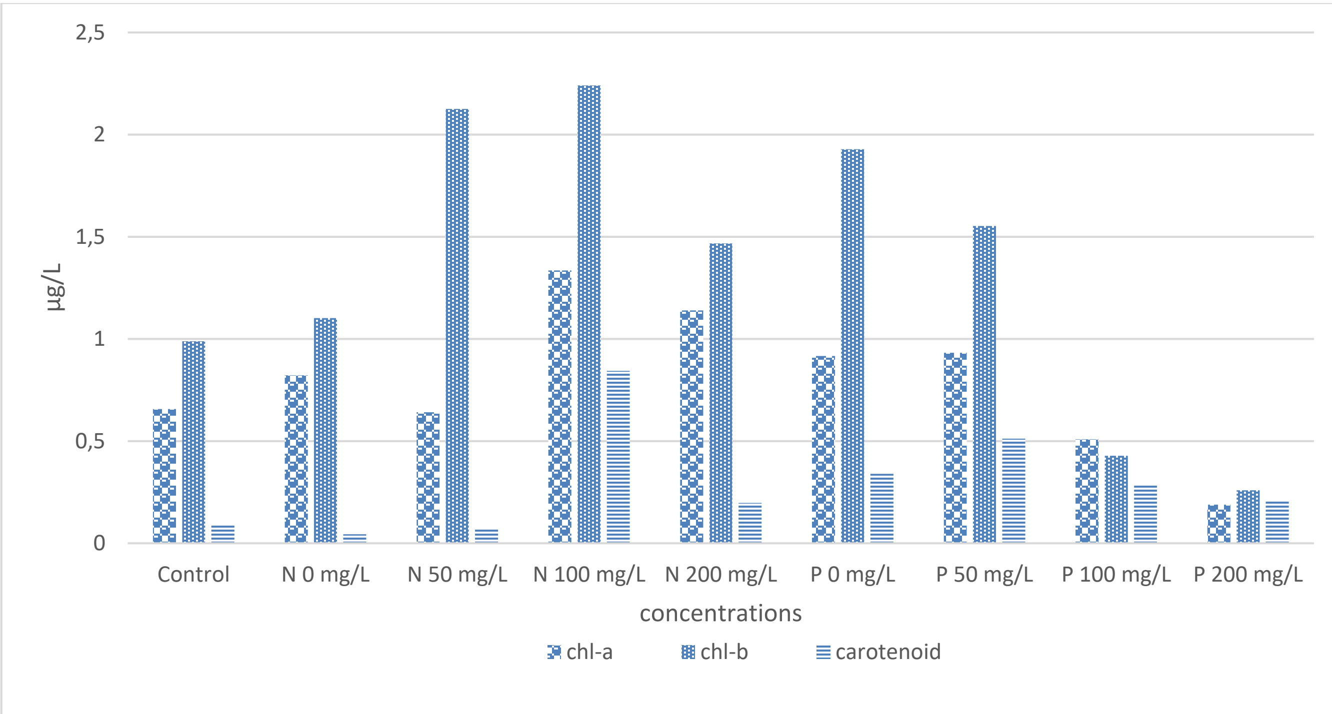

Figure 1. Chl-a and b and carotenoid changes in µg/L

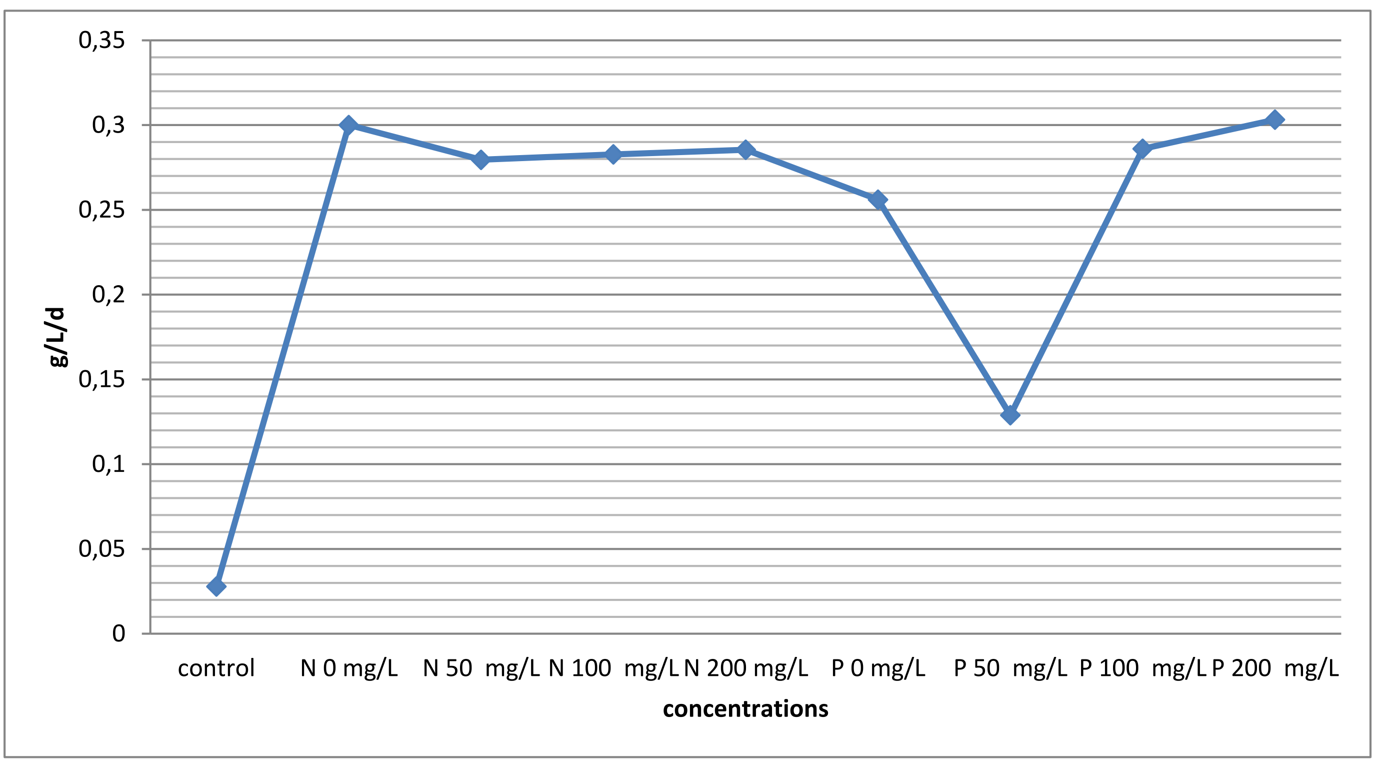

Figure 2. Carbohydrate content changes in g/L after the nutrient treatment

Total Carbohydrate Contents

The effects of high concentrations of nutrient on the carbohydrate content and productivity of *C. vulgaris* under mixotrophic cultivation can be seen in Figure 2. Carbohydrate content for the control group were measured 0.0278 g L^{-1} under mixotrophic culture conditions. Measurements for the carbohydrate content, for the 200 mg L^{-1} concentration of nitrate and phosphate solution, showed that the high concentration of NO_3^- and PO_4^{3-} in the culture media causing an increase in carbohydrate, respectively. The average carbohydrate content for nitrate and phosphate treatment measured as 0.2869 and 0.2435 g L^{-1}, showing that these nutrients cause an increase on the increasing concentrations.

Carbohydrates are found as the intermediary reserves in some algae, due to the fact that they are required when the nitrogen becomes limited in the lipid synthesis (Kong et al., 2013). In the present study, when chlorophyll content in *C. vulgaris* increased, both lipid and carbohydrate content increased by nitrogen depletion. A common trend can be since, in which the carbohydrate content increased rapidly after the nitrogen source concentration decreased to the lowest level, which is consistent with previous findings showing that carbohydrate accumulation in microalgae is often triggered by nitrogen depletion (Orus et al., 1991; Kong et al., 2013). These results suggested that changes in the cellular biochemical composition were influenced by the trophic conditions and nutrient concentration in the medium.

Result of Lipid Analysis

The measurements for the lipid content for the different nutrient concentration treatment showed that NO_3^- and PO_4^{3-} treatment causing an increase in lipid levels. The max. lipid content was 27.95 and 29.53 mg L^{-1} under nitrate and phosphate treatment medium, respectively (Figure 3). Woertz et al. (2009) studied the lipid productivity and nutrient removal by green algae including *Scenedesmus, Chlorella* and *Glolenkinia* species grown during the wastewater treatment in batch cultures and reported that the maximum lipid content range was 14-29% and volumetric productivity of lipid was 17 mg/L/d. The highest lipid content (30.74 and 39.88 mg L^{-1}) occurred in mixotrophic cultivation when the culture was loaded with a high concentration of nitrate and phosphate (100-200 mg L^{-1}), higher than under autotrophic cultivation.

The lipid productivity obtained in the present work was not necessarily superior or inferior to those reported elsewhere using different strains of microalgae. For instance, Converti et al. (2009) and Woertz et al. (2009) reported that *C. vulgaris* growing in Bold's basal medium had somewhat higher production rates ranging from 8 to 20 mg/d/L and 17 to 24 mg/d/L, respectively. This suggests that in laboratory culture mode the lipid productivity in wastewater or simulated wastewater might be improved by continuous supplementation of nutrients such as nitrate or phosphate (Wang, 2012).

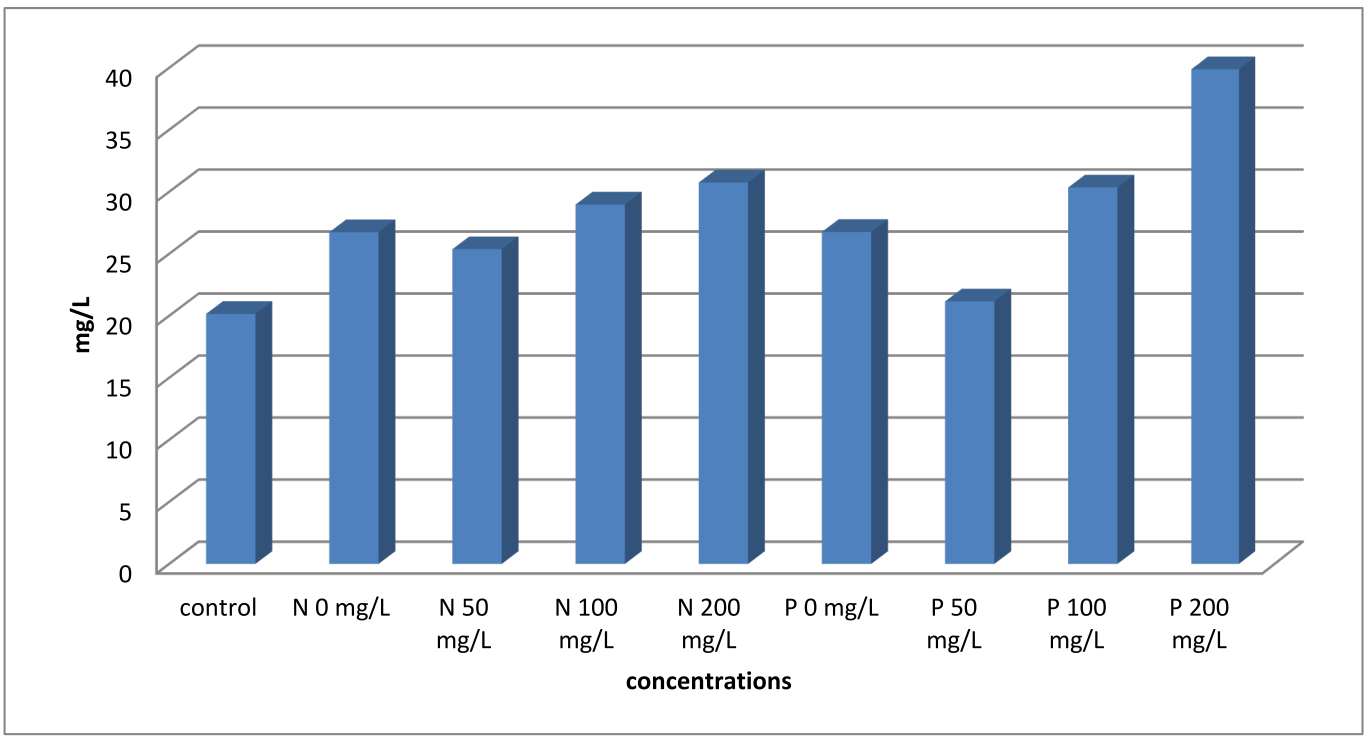

Figure 3. Lipid content changes in mg/L after the nutrient treatment

Nutrient Removal Efficiencies

The removal amounts and removal efficiency of total nitrogen and phosphorus depending on the four different concentration of culture medium are presented in Figures 4 and 5. The min. and max. nitrate removal amounts and efficiency were 0.2302- 0.3584 mg L^{-1} and 63.61- 99.05% under mixotrophic conditions, respectively. The results showed that the mixotrophic cultures experience an increase in nitrate uptake that was dependent on the increase of high concentration of nutrient in the medium content. The NO_3^- uptake capacities was average 88.29%. It means that mixotrophic microalgae approximately consumed about 89% of the initial nitrate after 10 days to produce biomass.

Max. phosphate removal amount and efficiency were also high, as great as 0.2226 mg L^{-1} and 63.77% in mixotrophic conditions compared to the other concentration of culture medium at 50 mg L^{-1} nitrate concentration of nutrient in the medium content. Lowest phosphate removal capacity was observed in 100 and 200 mg L^{-1} concentration of treatment.

This might be to the fact that the organic carbon concentrations in this experiment were low compared to those in the reviewed literature de-Bashan et al. 2011.

Under the mixotrophic and optimum conditions (200 µmol photon $m^{-2}s^{-1}$ 16 h photoperiod and 28% inoculum size), 63.61-99.05% of NO_3^- and 13.97-63.77% of PO_4^{3-}were successfully removed (Tab 2).

Mixotrophic cell cultivation utilizing both light and organic carbon source has been considered the most efficient process for the production of microalgal biomass (Lee et al., 1996). When the light energy used for CO_2 fixation is decreased in mixotrophic cultures, most of the energy is used for carbon assimilation. Therefore, since the amount of energy dissipated is minimal, mixotrophy provides higher energetic efficiency than other cultivation modes (Lalucat et al., 1984). On the other hand, Shi et al. (2000) reported that glucose can be considered the best organic C-substrate for the growth of *Chlorella.*

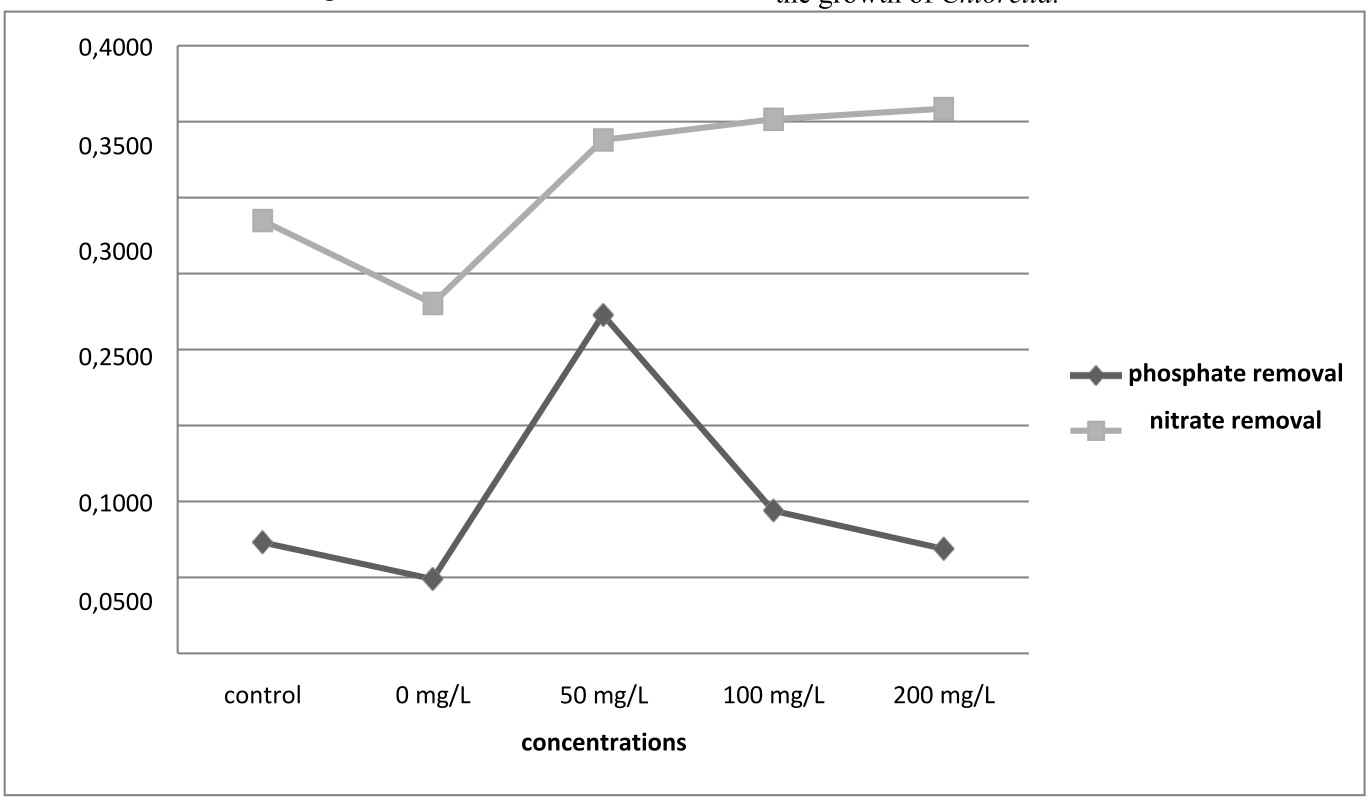

Figure 4. Nutrient removal levels measured for 0; 50; 100; 200 mg L^{-1} and control values.

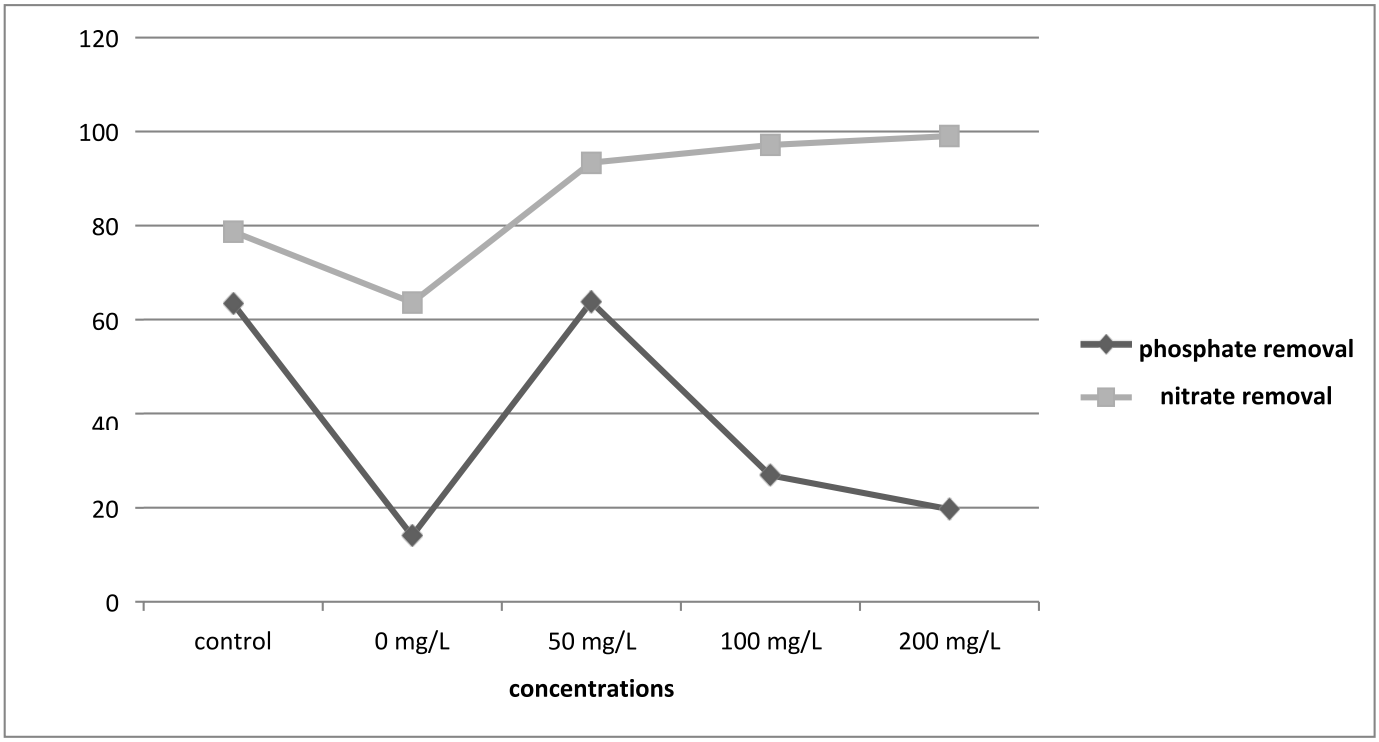

Figure 5. Nutrient removal efficiency of *C. vulgaris* under the mixotrophic conditions

Under the mixotrophic and optimum conditions (200 µmol photon $m^{-2}s^{-1}$ 16 h photoperiod and 28% inoculum size), 63.61-99.05% of NO_3^- and 13.97-63.77% of PO_4^{3-}were successfully removed (Tab 2).

Mixotrophic cell cultivation utilizing both light and organic carbon source has been considered the most efficient process for the production of microalgal biomass (Lee et al., 1996). When the light energy used for CO_2 fixation is decreased in mixotrophic cultures, most of the energy is used for carbon assimilation. Therefore, since the amount of energy dissipated is minimal, mixotrophy provides higher energetic efficiency than other cultivation modes (Lalucat et al., 1984). On the other hand, Shi et al. (2000) reported that glucose can be considered the best organic C-substrate for the growth of *Chlorella.*

Conclusion

To conclude, this study describes the nutrient removal efficiency of *C. vulgaris* under the mixotrophic conditions while illustrating the effect of high concentration of nitrate and phosphate solution on carbohydrate, chlorophyll and carotenoid content as well as its relation between lipid synthesis levels. The findings from the study show that the uptake of nutrient with *C. vulgaris* green microalgae for excess nitrogen and phosphorus removal is effective. Generally, *C. vulgaris* removed more nutrients from mixotrophic medium than the control medium. Uptake of nitrate by the culture was the highest under mixotrophic conditions than the autotrophic conditions (control medium) at 0, 50, 100 or 200 mg L^{-1} nutrient concentrations. Uptake of phosphate was higher under autotrophic conditions at 50 mg L^{-1} nutrient concentrations. It was concluded that the mixotrophic regime, using glucose, is superior to autotrophic regime for the uptake of nitrate. The activity of *C. vulgaris* microalgae on practical aqueous solution nutrient removal will reduce drastically the concentration of excess nitrogen that will be discharged into the various compartments of the environment, and can even find use in agricultural farms as irrigation water.

Table 2. Standardized conditions (control) and under high concentration of nitrate and phosphate treatment from the 10th day of mixotrophic culture condition. Values are expressed as amount of substances in relation to the dry matter. Each value represents the mean of three replicates ± standard deviation.

	Chl-a (µg/L)	Chl-b (µg/L)	Carotenoid (µg/L)	Carbohydrate (g/L)	Lipid (mg/L)	Adsorption capacities (mg/L)	Uptake efficiency (%)
Control	0.6565±0.006	0.9883±0.005	0.0945±0.004	0.027±0.004	20.15±0.3	0.1788±0.001	71.04±0.1
N 0 mg/L	0.82181±0.005	1.1019±0.007	0.0444±0.004	0.3000±0.004	26.73±0.9	0.2302±0.002	63.61±0.4
N 50 mg/L	0.6408±0.002	2.1255±0.006	0.0689±0.006	0.2795±0.005	25.36±0.2	0.3379±0.005	93.35±0.1
N 100 mg/L	1.3348±0.005	2.2404±0.004	0.8437±0.006	0.2827±0.006	28.96±0.9	0.3515±0.005	97.13±0.3
N 200 mg/L	1.1393±0.004	1.4674±0.005	0.1974±0.004	0.2854±0.002	30.74±0.7	0.3585±0.002	99.05±0.3
P 0 mg/L	0.9163±0.006	1.9277±0.006	0.3396±0.001	0.001±0.001	26.75±0.9	0.0488±0.003	13.97±0.5
P 50 mg/L	0.9317±0.004	1.5525±0.002	0.5120±0.001	0.1288±0.002	21.15±0.9	0.2227±0.004	63.76±0.3
P 100 mg/L	0.5087±0.004	0.4285±0.003	0.2891±0.006	0.2860±0.001	30.34±0.8	0.0938±0.004	26.85±0.6
P 200 mg/L	0.1890±0.005	0.2589±0.003	0.2096±0.005	0.3032±0.004	39.88±0.4	0.0686±0.003	19.65±0.1

Compliance with Ethical Standard

Conflict of interests: The authors declare that for this article they have no actual, potential or perceived conflict of interests.

Ethics committee approval: There is no need ethics committee approval.

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Evaluation of Spirulina (*Spirulina platensis*) wastes and live housefly (*Musca domestica*) larvae as dietary protein sources in diets of *Oreochromis niloticus* (Linnaeus 1758) fingerlings

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ABSTRACT

This study was designed to evaluate spirulina wastes and live housefly maggot as partial replacement for fishmeal in the diets of Nile Tilapia. Four isonitrogenous (35%) and isocaloric (17-18 $KJ.g^{-1}$) diets were evaluated: commercial diet Skretting SK, control diet CD (30% fishmeal), and two others diets (SW and LM), corresponding to spirulina wastes and live housefly maggot inclusion respectively. Diets were hand-fed thrice daily to triplicate groups of fish to apparent satiation. After 12 weeks, the final weight and feed intake of fish fed diet CD and SK did not differ from those fed diets LM, but were higher than those fed SW diet ($p<0.05$). However, inclusion of SW had no effect on feed utilization. No significant differences were found in survival, feed conversion ratio, protein efficiency ratio and condition factor among the treatments ($p>0.05$). Whole-body protein contents were similar in all groups, whereas the lipid content was lower in SW group. It was concluded that a 62.56 % fishmeal protein could be replaced by live maggot in the diet of Nile Tilapia without negative effects on growth performances and quality of fish produced.

Keywords: Spirulina wastes, Live maggots, Fishmeal, Growth, Nile tilapia

Introduction

Nowadays, aquaculture industry provides half of all fish for human consumption (FAO, 2016; Esmaeili *et al.,* 2017). The expansion of this sector highly depends on industrially feeds (Tacon *et al.,* 2006). This feed rely on fishmeal as a major source of protein, highly digestible essential amino acids and fatty acids (Cho and Kim, 2011). The use of fishmeal as a major protein source in fish feed has heavily pooled to increased demand and prices for this raw material. Finding a suitable substitute for fishmeal is one means to reduce total operating costs in aquaculture industry (Webster *et al.,* 1997). Furthermore, plants protein do not constitute the utmost alternatives to fishmeal, and for this reason, the need to find new aquafeed ingredients presently remains a real challenge (Vizcaino *et al.,* 2014).

In plant protein, microalgae has received significant consideration in fish feed manufacturing because of its high protein content, vitamins, polysaccharides, polyunsaturated fatty acids, microelements and antioxidant pigments (Hemaiswarya *et al.,* 2011). Among the microalgae, Spirulina, which is a quite promising source of protein, is widely distributed and easily cultured in tank (Huo *et al.,* 2012). In recent years, Spirulina meal has been successfully used as a feed additive (Silva-Neto *et al.,* 2012) or alternative protein source (Teimouri *et al.,* 2013; Velasquez *et al.,* 2016) in aquafeeds to improve weight gain and carcass quality of fish. Because *Spirulina platensis* is one of the most habitually used dietary complements in human comsumption and many animal species, including fish. Its wastes, which is rich in protein, can be used as dietary protein source in Nile Tilapia diets. Abdelkhalek *et al.* (2015) indicated that *Spirulina platensis* supplementation in *O. niloticus* diets, could minimize deltamethrin (DLM) induced toxic effects by its mighty antioxidant activity. It is also a protective agent anti hepatotoxicity in freshwater catfish *Clarias batrachus* (Ahmad Dar *et al.,* 2014). Inclusion of *Spirulina maxima* in diets for juvenile common carp *Cyprinus carpio* results in increased growth rate (Ramakrishnan *et al.,* 2008).

At the present time, some plant protein sources, such as Azolla meal (Abou *et al.,* 2007ab) ; cereal grain products and by-products (Guimarães *et al.,* 2008), corn co-products (Herath *et al.,* 2016), *Jatropha curcas* kernel meal (Krome *et al.,* 2016) ; soybean meal (Al-Feky *et al.,* 2016), have been used to partially or totally replacement fishmeal in diets of Nile Tilapia. However, greatest in amount plant-based feedstuffs have a large variety of anti-nutritional factors, which may decrease fish growth performance. To ensure high production and fast growth at least cost, a well-balanced formulated feed is necessary for profitable tilapia farming. In some countries, different by-products such as chicken viscera are frequently left to rot in environment. This by-product pose pollution and health problems to local communities. Moreover, the poultry production industry generates large amounts of by-products (Adler *et al.,* 2014). However, there is currently poorly used as a protein source in aquafeeds. We can used this by-products to produce enriched housefly maggot.

The housefly (*Musca domestica*) (Diptera : Muscidae) can feed on a wide variety of spoiled organic matter, such as distillers grains, fish offal, food and vegetable waste and animal manure (Salomone *et al.,* 2017). In addition, insect's larvae have the potential to convert the animal manure into precious biomass. For instance, blacks soldiers flies has been assessing as a prospective animal to use in bioconversion of manure to reduce waste remnant. They can reduce nitrogen waste by 75% and mitigate mass by 50% in poultry process (Newton *et al.,* 2005). As mentioned above, chicken viscera poses a potential feedstock for housefly larvae. Housefly maggots are rich in proteins and lipids, and research on their use as meals has given good results for several of the aquaculture species tested (Ogunji *et al.,* 2008; Lin and Mui, 2016). Although, several studies on maggot meal have been published (Ogunji *et al.,* 2008 ; Wang *et al.,* 2017), little reports have been performed about the use of live housefly ; this is case of African catfish (Emeka and Oscar, 2016) and no reports in Nile tilapia, the most important farmed tilapia species around the world. Thus, use of live housefly maggot for *O. niloticus* diets as fishmeal replacement is warranted. For this purpose, our study was aimed to assess a animal protein source (live housefly *Musca domestica* maggot) and single-cell protein spirulina *Spirulina platensis* wastes, tested separately, in practical diets for Nile tilapia substituting the fishmeal component in formulated experimental diets for this species.

Material and Methods

Fish and Experimental Procedures

Monosex male Nile Tilapia fingerling (*O. niloticus*) were obtained from Private fish farming ''Dieu Exauce'' located in Tori Avamey at Tori-Bossito (Benin). Tilapia were transported in oxygenated plastic bags to the Experimental Fish Farming Unit of Laboratory of Ecology of Aquatics Ecosystems of the University of Abomey Calavi, Benin, where the experiment was realized. Initially, 350 fish were stocked in $1m^3$ circular concrete tank and maintained during one week before start the feeding trial. During this time, they were fed with a mixture of experimental diets. A total of 600 fish with an average weight 8.65 ± 0.5 g were equally distributed into four experimental triplicate groups and stocked into 12 circular concrete tanks (diameter : 120 cm with capacity of 1000 l). Before the beginning of the experiment, fish were starved for 24 hours. The fish were fed their assigned diets thrice a day (09 : 00 ; 13 :00 and 17 :00 h) to apparent satiation and the quantity of feed consumed recorded for each tank. A outdoor recirculation rearing system was used to conduct the experiment, with water flow set at 3 L min^{-1}. Fish were weighed collectively at the beginning and fortnightly for each tank to determine gain in weight.

Ingredients and Experimental Diet

Housefly *Musca domestica* larvae produced from chicken viscera and spirulina *Spirulina platensis* wastes were used as a partial protein replacement of fishmeal in fish diets. Chicken viscera were collected from the poultry processing industry "Agrisatch" (Abomey-calavi, Benin), and incubated in an rectangular areas (measuring 3m x 2m) as a substrate for housefly larvae development. The substrate was watered twice daily with water to prevent drying and exposed for two days to let houseflies to spawn eggs on it. The substrate was covered and left among 3 to 5 days to enable maggot to be grown before harvesting. The harvested houseflies maggots were washed and pre-cooked in warm water at 85°C during 15 minutes in order to prevent disease pathogens infection, before being incorporated in the practical diet.

Sardinella sp fishmeal was used in the formulation of experimental diets. This ingredient is purchased at the Dantokpa market and sun-dried for three days before being transformed into meal. Blood meal was obtained following the procedures described by Alofa *et al.* (2016). The rest of the ingredients for the diets such as soybean meal, cottonseed meal, palm oil and salt were obtained at local market. Dry matter, crude protein, ether extracts and ash of housefly maggot and spirulina wastes used in this experiment were analysed (Table 1) to assist in experimental diet formulation (Table 2). The costs of ingredients used in the formulation of practical diets are given in Table 3.

After 2 week of acclimatization, the fish were fed one of the four experimental diets (3 tanks per treatment) for 86 days : one commercial diet Skretting SK, one control diet CD (no housefly maggot and spirulina wastes), and diets to which 15 % and 25 % of spirulina wastes and live housefly maggot were added respectively. Diets were denoted LM (250 $g.Kg^{-1}$ live maggot ; 935 g.Kg basis live weight) ; SW (150$g.Kg^{-1}$ Spirulina wastes). Spirulina wastes was supplied by Spirulina Production Unit of the Regional Institute for Development and Health (SPU/RIDH), located in Pahou (Ouidah, Benin). These wastes were generated from the production and packaging process of spirulina.

Preparation

Diets were formulated to contain 35 % crude protein and 17-18 kJ.GE g^{-1} diet (Table1). Ingredients were grounded, weighed, and mixed. Mixtures were then pelleted using a meat grinder to form pellets. The pellets were sun-dried and stored in plastic bags at - 4 °C until use. For the preparation of diet containing live maggot, this by-product was precooked ground with food grinder (Binatone BLG 450) and blended at least to make a paste before being to added to others ingredients.

Sampling and Water Quality Monitoring

Twenty fish were randomly selected to determine initial whole fish body nutrient composition and stored at -20°C until analysis. Biomass of each tank was recorded at the beginning and end of this trial. Ten fish per tank were randomly chosen (n = 30 per treatment). Fish weight, total length, were recorded to calculate condition factor (CF).

Water parameters such as hydrogen potential (pH), temperature (°C), dissolved oxygen (mg/L), salinity (*psu*), conductivity (µS/cm) and total dissolved solid (TDS mg/L) were measured weekly at a deep of 10 cm for each reared tank with a multiparameter probe (Hanna HI 9829 v1.04, Hanna Instruments Ltd., USA). Nitrite and ammonium were determined by cadmium reduction and phenate methods respectively using spectrophotometer Hach DR6000. These parameters were checked three times fortnightly.

Calculations

To show the effect of spirulina wastes and live housefly larvae inclusion on growth performance and nutritional indices, the next parameters were determined as average of the triplicates by the formulas given.

$$\textbf{Survival rate (SR, \%)} = \frac{\text{final amount of fish}}{\text{initial amount of fish}} \text{ X } 100$$

$$\textbf{Weight gain rate (WGR, \%)} = \frac{(\text{final body weight} - \text{initial body weight})}{\text{initial amount of fish}} \text{ X } 100$$

$$\textbf{Specific growth rate (SGR, \%)} = \frac{Ln(final\ weight\ gain) - \ln(\text{initial weight})}{\text{rearing period}} \text{ X } 100$$

$$\textbf{Feed intake (FI, g/fish)} = \frac{\text{total amount of the dry feed consumed}}{\text{fish numbers X days}} X\ 100$$

$$\textbf{Protein efficiency ratio (PER)} = \frac{\text{body weight gain}}{\text{total feed consumed protein content in diets}}$$

$$\textbf{Feed Conversion Ratio (FCR)} = \frac{\text{total dry feed consumed}}{\text{body weight gain}}$$

$$\textbf{Condition factor (CF)} = \frac{\text{final body weight (g)}}{\text{body length (cm)3}} X\ 100$$

$$\textbf{Yield (Kg/m3)} = \frac{\text{final biomass per tank (g)} - \text{initial bimass per tank (g)}}{\text{water volume (1 m3)}}$$

$$\textbf{Production (Kg/m3/year)} = \frac{\text{Yield x365}}{\text{rearing period}}$$

Economic conversion ratio (ECR)

= Cost of diet x Feed Conversion Ratio (FCR)

$$\textbf{Profit index (PI)} = \frac{\text{Price of fish produced}}{\text{Price of feed consumed}}$$

Table 1. Formulation and proximate composition of experimental diets fed monosex Nile Tilapia fingerlings during 12 week

	Dietary treatments			
	SK[1]	CD	SW	LM
Ingrédients **(g 100 g $^{-1}$)**				
Sardinella sp. **fishmeal**		30	10	10
Spirulina wastes		–	15	–
Live housefly maggot		–	–	25
Blood meal		7	7	7
Corn bran		36	21	25
Soybean meal		14	25	22
Cottonseed meal		10	19	11
Palm oil		2	2	2
Salt (NaCl)		1	1	1
Proximate composition				
Dry matter (%)		90.16	90.31	90.24
Crude protein (% DM)		35.32	35.08	35.13
Crude lipid (% DM)		8.15	9.19	11.88
NFE[2]		36.42	34.15	31.95
Ash (% DM)		7.95	7.21	6.45
Gross energy[3] (kJ g $^{-1}$)		17.85	17.82	18.58
Diet cost (US\$. Kg $^{-1}$)[4]	1.87	1.00	0.69	0.67

1. Proximate composition : Crude protein : 35% ; Crude fat : 9% ; Fibre : 3.4% ; Ash : 6.5%, Calcium : 1% ; Phosphore : 1%, Lysine : 1.5% ; Methionine : 0.5% ; CuSO4 : 5mg/Kg
2. Nitrogen-free-extract (NFE) = 100 - (% moisture + % crude protein + % crude lipid + % ash + % crude fibre).
3. Gross energy (GE) was calculated using the factors of 23.7 KJg $^{-1}$, 39.5 KJg $^{-1}$ and 17.2 KJg $^{-1}$ protein, lipids and carbohydrates respectively (Guillaume *et al.,* 1999).
4. Prices in US$, 1 US$= 586.69 FCA at present. Including labour and processing

Table 2. Analysed nutrient composition (as % dry matter) of feeds ingredients

Ingredients	Dry matter	Crude protein	Crude lipid	Ash	Crude fibre
Fish meal	92.0	66.0	7.88	15.77	1.0
Spirulina wastes	91.53	46.32	6.71	10	3.2
Maggot meal	91.7	48.8	20.1	6.25	6.1
Soybean oilcake	94.8	30	13.2	3.7	6.0
Cottonseed oilcake	90.0	40.5	7.0	8.0	14.0
Blood meal	90.9	71.9	1.7	6.4	1.6
Maize bran	91.4	6.2	3.1	1.4	12.3

Proximate Analysis

Dry matter, crude protein, crude lipid, and ash in feed ingredients and fish (Table 1) were determined according to standard procedures of Association of Official Analytical Chemists, AOAC (Horwitz and Latimer, 2005). Dry matter was determined by drying samples in an oven (Memmert UN160 Plus) at 105°C for 24 hr. Crude protein was calculated from the nitrogen content (N x 6.25) using the Kjeldahl method. Samples were first acid-digested. Crude lipid content in samples was determined by chloroform–methanol method (Folch *et al.,* 1957), while crude ash content was determined by incineration samples at 500°C for 12 h and weighing the residual ash. All analysis was performed in triplicate.

Statistical Analysis

Data were expressed as the mean ± SEM. of triplicate samples. All statistical analyses were conducted using Microsoft Excel and Statistical Package for Social Sciences (SPSS IBM version 20.0 for windows v8.1, Chicago, Illinois, USA). Prior analysis, homogeneity of variance was determined using the Hartley statistical test after log transforming (Dagnelie, 1975). Differences in the mean levels of the parameters between the dietary treatments were determined using one way analysis of variance ANOVA followed by Tukey's test of multiple comparison. The differences were considered significant when p-value were < 0.05.

Results and Discussion

The search for sustainable ingredients to replace fishmeal has been a real challenge for the Tilapia industry. At fishmeal substitution experiment, the quality of FM is of great importance on how tested products perform as FM substitutes (Biswas *et al.,* 2017). In this experiment, a high quality of FM produced from *Sardinella spp* with protein contents approximately 660 g kg^{-1} was used. Housefly larvae are converters of organic waste into expendable biomass of which the composition may attribute on the substrate. In this experiment, larvae were grown on chicken viscera.

The effects of SW and LM inclusion on tilapia performance, nutrient utilization and production are presented in Table 5. Although there was little variation in lipid contents as indicated in Table 1, all experimental diets were isocaloric and isonitrogeneous. Growth parameters were poor in fish fed SW diet and similar ($p < 0.05$) in those fed CD, SK and LM diets (Figure 1). There were no significant differences ($p > 0.05$) in final weight (80.96 – 88.54 g), DWG (0.86 – 0.95 g. $days^{-1}$), SGR (2.68 – 2.77 % $days^{-1}$) and annual production (13.88 - 16.03 Kg/m^3/ year) of Nile tilapia fed with control diets and LM diet (Table 5). These findings indicated that the growth performance and feed efficiency of *O. niloticus* juveniles fed live housefly larvae were not significantly affected by the replacement of fishmeal up to 66 %, showing that LM protein can be used to partially substitute FM in a practical diet of Nile tilapia. This is in agreement with the findings of Oyelese (2007) and Ogunji *et al.* (2008) that used it as partial FM substitute without affecting growth and feed utilization in Tilapia and catfish juveniles. The current study is in agreement with the earlier reported Tilapia studies and exemplifies the possible use of live housefly maggot as a partial substitute for FM in *O. niloticus* diets. Studies evaluating live housefly larvae in fish diets are highly few, but rising. Results of the current study in Nile Tilapia are similar to several studies in Teleost. In rainbow trout (*Oncohynchus mykiss*) for example, fish fed a diet with 18 up to 36% maggot meal (MM) produced from cow manures and fish offal had similar final average weight and weight gain as fish fed a control diet, whereas fish fed a diet containing 16 up to 33 % MM produced from cow manures only had significantly reduced growth parameters (Sealey *et al.,* 2011). This may be presumed that the nutrient content of fly larvae largely depends on their diet (Spranghers *et al.,* 2017). In the present experiment, specific growth rate recorded in all traitment were comparatively higher than those of the anterior study (Wang *et al.,* 2017) in which the SGR of Nile tilapia (initial weight : 68.89 g) fed housefly MM were ranged from 1.12 to 1.62 % per day. This difference might be due to the fish sizes or further rearing conditions.

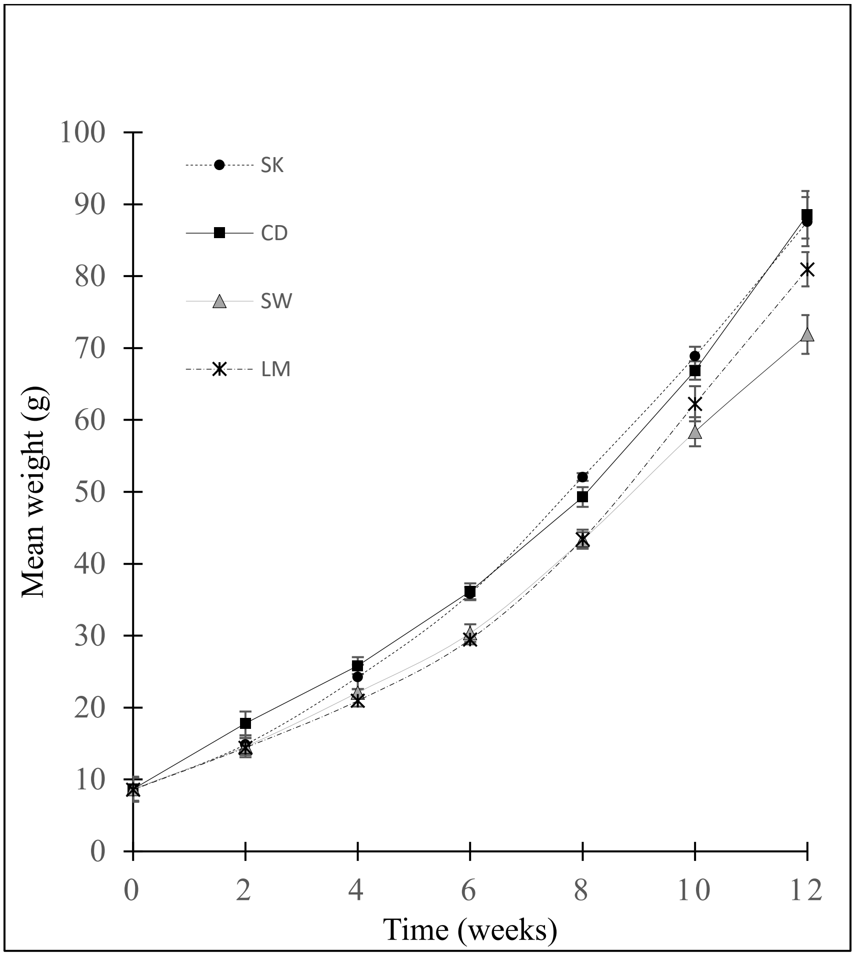

Figure 1. Mean weight (g) evolution of juvenile Nile Tilapia *Oreochromis niloticus* fed the commercial tilapia diet Skretting SK, the experimental diets containing *Sardinella sp.* fishmeal CD, Spirulina wastes SW and live housefly maggot LM during 12 weeks.

Table 3. Cost of ingredients used in formulating the diets

Ingredients	Price (US$.Kg^{-1})
***Sardinella sp* fishmeal**	2.24
Soybean meal	0.67
Cottonseed meal	0.33
Blood meal	0.22
Spirulina wastes meal	0.43
Housefly maggot meal	0.44
Corn bran	0.26
Palm oil	1.38
Salt (NaCl)	0.43

Table 4. Water quality parameters in *O. niloticus* rearing tanks during the experimental period

Parameters	SK	CD	SW	LM
pH	6.78 ± 0.29	6.81 ± 0.29	6.85 ± 0.33	6.83 ± 0.30
Temperature (°C)	29.88 ± 0.68	30.08 ± 0.72	29.88 ± 0.78	30.27 ± 1.47
Dissolved oxygen (mg. L^{-1})	3.12 ± 0.56	3.17 ± 0.57	3.15 ± 0.58	3.08 ± 0.31
Conductivity (µS/cm)	179.7 ± 84.1	185.1 ± 86.9	181.8 ± 88.0	183.0 ± 82.5
TDS (mg. L^{-1})	93.06 ± 45.56	94.63 ± 45.22	93.35 ± 45.67	93.31 ± 42.29
Salinity (psu)	0.07 ± 0.04	0.08 ± 0.04	0.08 ± 0.04	0.08 ± 0.04
Nitrate (mg. L^{-1})	2.23 ± 0.38	2.33 ± 0.25	2.27 ± 0.15	2.73 ± 0.35
Nitrite (mg. L^{-1})	0.04 ± 0.00	0.03 ± 0.01	0.03 ± 0.01	0.03 ± 0.01

Data are expressed as means ±SE (n = 3)
SK: Skretting, CD: control diet, SW: spirulina wastes diet, LM: live housefly maggot diet

Table 5. Growth performance, feed efficiency and annual production of *Oreochromis niloticus* fed the experimental diets for 12 weeks.

Parameters	SK	CD	SW	LM
Initial weight (g)	8.60 ± 0.10	8.66 ± 0.14	8.68 ± 0.14	8.53 ± 0.03
Final weight (g)	87.59 ± 3.42^{a}	88.54 ± 3.30^{a}	71.89 ± 2.70^{b}	80.96 ± 2.38^{a}
Feed intake (g $fish^{-1}$)	94.93 ± 3.20^{ab}	104.84 ± 3.33^{a}	83.73 ±3.17^{c}	94.81 ± 4.54^{bc}
Survival rate (%)	96.00 ± 2.00	94.00 ± 2.00	94.00 ± 2.00	91.33 ± 2.31
Weight gain (%)	918.8 ± 41.8^{a}	923.4 ± 54.7^{a}	728.8 ± 31.2^{b}	848.8 ± 30.8^{a}
Daily weight gain (g. $days^{-1}$)	0.94 ± 0.04^{a}	0.95 ± 0.04^{a}	0.75 ± 0.03^{b}	0.86 ± 0.03^{a}
Specific growth rate (% $days^{-1}$)	2.76 ± 0.05^{a}	2.77 ± 0.06^{a}	2.52 ± 0.04^{b}	2.68 ± 0.04^{a}
Feed conversion ratio	1.21 ± 0.09	1.32 ± 0.02	1.34 ± 0.02	1.32 ± 0.05
Protein efficiency ratio	2.37 ± 0.18	2.16 ± 0.04	2.14 ± 0.03	2.16 ± 0.09
Condition factor	1.92 ± 0.09	1.89 ± 0.12	1.80 ± 0.02	1.80 ± 0.07
Yield (Kg/m^3)	3.78 ± 0.25^{a}	3.73 ± 0.25^{a}	2.94 ± 0.07^{b}	3.27 ± 0.17^{ab}
Production (Kg/m^3/ year)	16.03 ± 1.06^{a}	15.84 ± 1.06^{a}	12.49 ± 0.30^{b}	13.88 ± 0.71^{ab}

Values in a row with different letters are significantly different ($p < 0.05$, Tukey's test).
SK: Skretting, CD: control diet, SW: spirulina wastes diet, LM: live housefly maggot diet

Water quality parameters values during the feeding trial were exposed in Table 4. The water temperature ranged from 29.88 to 30.27 °C, pH from 6.78 to 6.85, dissolved oxygen from 3.08 to 3.17 mg.L^{-1} , salinity from 0.07 to 0.08 mg.L^{-1}, nitrate from 2.23 to 2.73 mg.L^{-1} and nitrite from 0.03 to 0.04 mg.L^{-1}. No significant differences were observed in these parameters ($p>0.05$). These parameters recorded were optimal for the monosex male Nile Tilapia used in the experiment, because the optimal temperature for this species must be in a range between 12 and 16ºC and the dissolved oxygen content should not lower than 3 mg. L^{-1} (Bhujel, 2000). Thereby, experimental diet did not affect significantly the pH of the dietary traitment, these remarks were in contrariness of the results noted from Promya and Chitmanat (2011) that recorded higher values of pH in tank with fish fed with a diet including algae. Survival rate of experimental fish were not affected by the presence of spirulina wastes in the diet, ranging from 91 to 96%. Similar data were observed for several fish fed spirulina meal diets (Sirakov *et al.,* 2012; Promya and Chitmanat, 2011).

On the other hand, spirulina contains a large amount in proteins essential, vitamins, minerals, amino acids and fatty acids, antioxidant pigments and has been identified as a feed ingredient for cichlids; it seems to be a hopeful dietary protein source (Guroy *et al.,* 2012).

Feed intake had decreased ($p < 0.05$) in fish fed SW diet (83.73 ± 3.17 g. $fish^{-1}$). In contrast, fish fed LM diet (107.95 ± 3.31 g. $fish^{-1}$) had similar FI with those fed control diet SK and CD (94.93 ± 3.20 and 104.84 ± 3.33 g fish^{-1} respectively). Our results were in contrast of the data received from Guroy *et al.* (2012), who showed that Spirulina meal has the potential to enhance the growth, reproductive performance and coloration on yellow tail cichlid *Pseudotropheus acei*. Several studies have shown that dietary Spirulina can affect the growth performance of diverse fish species. For example, it has previously been reported that 20 up to 40% of FM can be substitute with spirulina meal without negative effect on the growth performance of hybrid red tilapia (Ungsethaphand *et al.,* 2010). Moreover, Guroy *et al.* (2012) reported that spirulina meal could be replaced fishmeal up to 10% in yellow tail cichlid diets without any adverse effects on growth, reproductive performance or coloration. Likewise, it has been reported that that dietary inclusion of 8% Spirulina significantly enhanced growth performance of the ornamental red swordtail *Xiphophorus helleri* (James *et al.,*2006). Furthermore, according to Yeganeh *et al.* (2015), it's well known that the increase in HDL-cholesterol with spirulina inclusion suggests that Spirulina may improve the cardiovascular activity in rainbow trout (*Oncorhynchus mykiss*).

No significant differences in feed conversion ratio (1.21 - 1.34), protein efficiency ratio (2.14 - 2.37) and condition factor (1.80 - 1.92) were observed among these groups. However, fish fed SW diet showed significantly lower final mean body weight compared with other group being represented the lower value ($p < 0.05$, Table 5). The poor growth performance in fish fed SW diet might be due to the lower digestibility of microalgae, due to the presence of a cellular wall, as suggested Le Vay *et al.* (2001). It has been demonstrated in most studies that low growth rates of fish fed with plant protein–based diets were attributed with poor feed intake that was strongly influenced by the palatability of diets (Kader and Koshio, 2012). In the present study, FI was significantly decreased in fish fed SW diet which indicated that SW protein sources can negatively affect palatability. FI is highly influenced by the palatability of diets; it's one of the most important factor coupled with the efficiency on the utilization of protein sources in fish (Kader *et al.,* 2012). Plant protein are successfully used in feed formulations for rearing tilapia species because Tilapias have herbivorous or omnivorous feeding habits and lower level of the aquatic food chain. Likewise, growth performances obtained with the spirulina wastes protein in this study was lower than previously reported for others aquatic species of similar weight fed with spirulina such as the yellow tail cichlid *Pseudotropheus acei* (Guroy *et al.,* 2012), *Litopenaeus schmitti* larvae (Jaime-Ceballos *et al.,* 2006), the sturgeon *Acipenser baeri* (Palmegiano *et al.,* 2005) and *O. mossambicus* X *O. niloticus* (Ungsethaphand *et al.,* 2010). It has been demonstrated that high quality spirulina meal was an adequate and nutritious protein source that increased growth in several species such as Common carp *Cyprinus carpio* (Ramakrishnan *et al.,* 2008) ; sturgeon *Acipenser baeri* (Palmegiano *et al.,* 2005). However, Spirulina meal has high protein content (i.e. 66.9 %) compared with spirulina wastes (46.32 % crude protein) used in the present study. Therefore, the adverse effect following SW inclusion might be also due to the lower protein content observed in fish fed SW diet.

In our study, PER was more favorable in spirulina wastes based diet than in the control diet SK and CD. A similar observation was made using *Spirulina platensis* at different levels in sturgeon *(Acipenser baeri)* (Palmegiano *et al.,* 2005). However, The FCR and PER in *O. niloticus* fed Spirulina wastes based diets were similar than those of the control diets CD and SK. The results of this work are similar to those found by Teimouri *et al.* (2013) in which rainbow trout (*Oncorhynchus mykiss*) fingerlings fed with control diet, 7.5 and 10% *S. platensis* inclusion diets as feed supplement, showed comparable feed conversion ratio. Furthermore, the cellular structure (mucopolymer murein) of Spirulina alga is readily digestible and does not contain cellulose (Beresto, 2001). Wherefore, significant decrease on growth performance in fish fed SW diet may be associated by the lower feed intake observed in these fishes.

The variation in the final whole-body proximate composition is reported in Table 6. Except dry matter and crude protein content, all whole body compositions were significantly affected by dietary protein source ($p < 0.05$). Crude lipid content in fish fed with SW diet were significantly lower than those in fish fed any other diets ($p < 0.05$). However, lipid contents was significantly higher in fish fed with LM diet, whereas ash content significantly decreased ($p<0.05$), reflecting the lipid and ash contents of this protein source. The results of the economic analysis are shown in Table 7. As it can be seen, profit index significantly increased with fish fed both SW and LM diets whereas economic conversion ratio decreased significantly. Economic analysis shows that inclusion of both SW and LM in the diet improves profitability.

Table 6. Proximate composition (%) of whole body of *Oreochromis niloticus* fed the experimental diets: CD, diet containing fish meal; LM, diet containing live housefly maggot and SW, diet with spirulina wastes meal.

Diets	Initial	SK	CD	SW	LM	p-values
Dry matter	89,82 ± 0,12	91,91 ± 0,04	90,50 ± 0,47	90,57 ± 0,06	91,45 ± 1,46	0,319
Crude protein	63,14 ± 0,70	61,40 ± 0,44	62,45 ± 0,08	60,26 ± 1,37	59,27 ± 1,13	0,090
Crude lipid	10,76 ± 0,59	32,59 ± 1,86[a]	33,56 ± 1,66[a]	26,15 ± 0,38[b]	35,77 ± 0,59[a]	0.007
Ash	16,52 ± 81,19	14,79 ± 0,68[ab]	17,43 ± 2,37[a]	15,29 ± 0,17[ab]	11.13 ± 1.32[b]	0,046

Values in the same column with different superscripted small letters mean significant difference ($p < 0.05$). Values show mean ± standard error, $n = 3$

Table 7. Summary of cost benefit analysis of Nile Tilapia fed the test diets

Diets	SK	CD	SW	LM	Anova p-values
Parameters					
Total feed used (Kg m^{-3})	4.56 ± 0.07[ab]	4.93 ± 0.26[a]	3.93 ± 0.12c	4.33 ± 0.32[bc]	0.003
Cost of feeding (US$.m^{-3})	8.517 ± 0.12[a]	4.93 ± 0.26[b]	2.72 ± 0.08[c]	2.90 ± 0.21[c]	0.000
Price of fish produced (US$.m^{-3})	9.78 ± 0.65[a]	9.66 ± 0.65[a]	7.62 ± 0.18[ab]	8.47 ± 0.43[b]	0.003
Economic Conversion Ratio (US$. Kg^{-1})	2.26 ± 0.18[a]	1.32 ± 0.02[b]	0.92 ± 0.02[c]	0.89 ± 0.04[c]	0.000
Profit Index	1.15 ± 0.09[c]	1.96 ± 0.03[b]	2.81 ± 0.05[a]	2.92 ± 0.12[a]	0.000

SK: Skretting, CD: control diet, SW: spirulina wastes diet, LM: live housefly maggot diet

Conclusion

In conclusion, the results clearly indicate that *O. niloticus* fed housefly larvae performed better than those fed spirulina wastes diet in terms of growth performance and feed utilization. Thus, 20% of FM could be saved by including 25 % of live housefly larvae in the diet of Nile tilapia without any adverse effects on the growth performance and feed utilization. In this study there is no supplement aminoacids, feed stimulants or other marine fish products, which also have concern over their future availability like to FM. This ensures the plainness of diet formula for the successful production of this species in rural areas.

Compliance with Ethical Standard

Conflict of interests: The authors declare that for this article they have no actual, potential or perceived conflict of interests.

Ethics committee approval: This study was conducted in accordance with ethics committee procedures of animal experiments.

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AQUATIC RESEARCH

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First record of *Fusinus dimassai* Buzzurro & Russo, 2007 (Gastropoda, Fasciolariidae) outside of the type locality: a further confirm of strict affinities between Messina strait and Tyrrhenian seamounts

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ABSTRACT

A revision of mollusc samples deposited in the Benthic Ecology Laboratory collections of Messina University, provided specimens of *Fusinus dimassai* Buzzurro & Russo, 2007, from the "Apollo Bank", south-western Tyrrhenian Sea. The record of such species, known so far from the Strait of Messina only, replicates the recent case of *Jujubinus errinae* Smriglio, Mariottini & Giacobbe, 2016, underlining the strict ecological affinities linking such peculiar area to the Tyrrhenian seamounts.

Keywords: Mollusc, Mediterranean, Laminariales, Biogenic seafloor, Biogeography

Introduction

The Strait of Messina, recognized as a peculiar "micro-sector", biogeographically distinct from all other Mediterranean area (Bianchi et al., 2012), is known as locus typicus of several mollusc species, as the spindle shell *Fusinus dimassai* Buzzurro & Russo, 2007. Such species, known so far only from the type locality, has been probably attributed in the past to the close *F. parvulus*, (Monterosato, 1884), together with other recently described congenerics (Russo, 2019). This uncertainty about the determinations preceding the description of the new species suggested a careful revision of material deposited at the Benthic Ecology Laboratory collections, Messina University. The revision led to the recognition of several specimens of *F. dimassai*, mostly from sampling carried out in the Strait of Messina, but also from the Isle of Ustica, south-western Tyrrhenian Sea.

Aim of this paper is to report the first finding outside of the type locality of this poorly known species, as a further evidence of the strict ecological affinities linking the Strait of Messina with the Tyrrhenian seamounts.

Material and Methods

The whole death assemblage described in Di Geronimo et al. (1988) has been revised, with particular focus on the genus *Fusinus*. The assemblage was associated to coarse sediments sampled from the "Apollo Bank" kelp beds, 3 km south-west of the Ustica volcanic system. The bank, ranging in depth from -40 m to -150 m (Figure 1), is characterized by rocky floors, irregularly covered by biogenic deposits, hosting dense populations of the Mediterranean gorgonian *Paramuricea clavata* (Risso, 1826) and, deeper, kelp beds of *Laminaria rodriguezii* Bornet 1888. The hydrology of the whole volcanic system, directly exposed to a branch of the Surface Atlantic Waters, is characterized by strong currents and upwelling.

The examined assemblage, dredged from 70 m to 50 m depth, has been originally sorted by a 7 dm^3 sediment sample, sieved onboard on a 1 mm mesh sieve. Each *F. dimassai* specimen, once separated from the original sample, has been photographed, examined, measured, and deposited apart (repository code: BEL138BA1988Fd1-6).

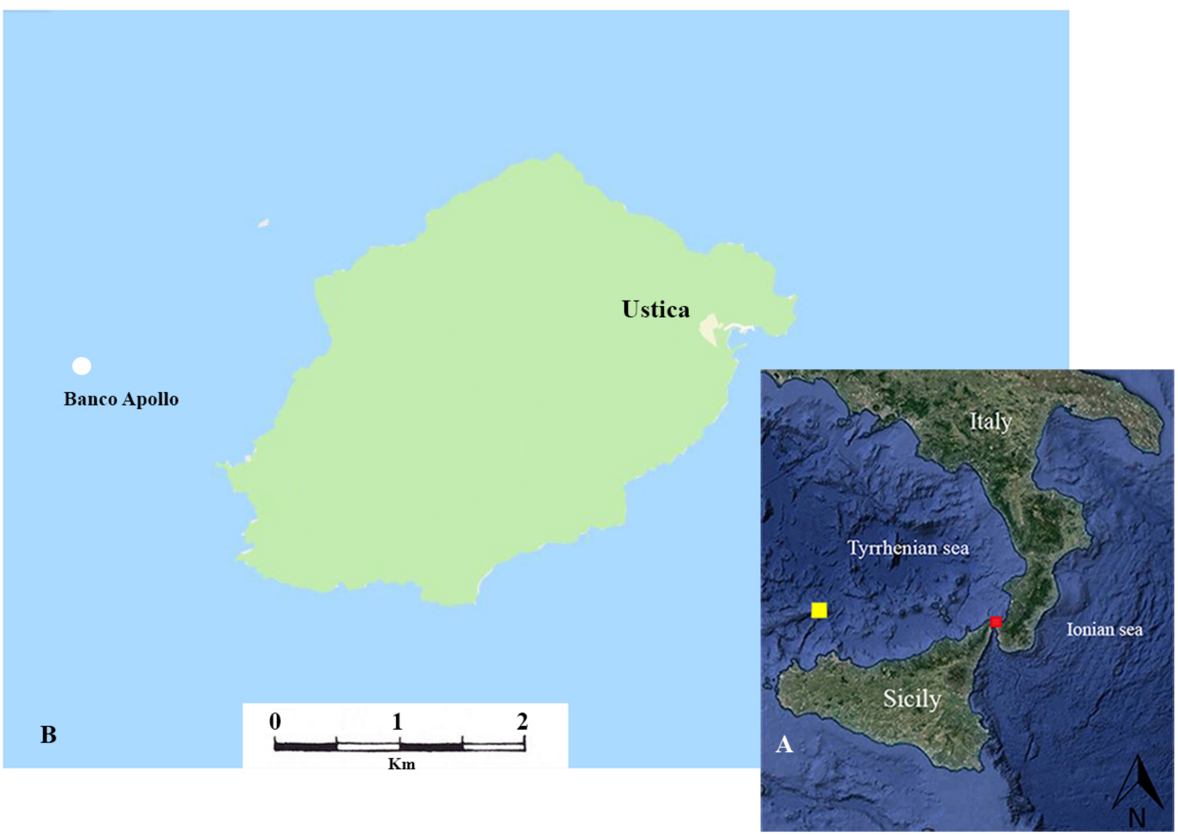

Figure 1. A) Sampling sites of *Fusinus dimassai*: the Strait of Messina (red circle) and the Isle of Ustica (yellow square) are indicated. B) Location of the Apollo Bank in the Ustica volcanic system.

Results and Discussion

The revision of the Apollo Bank death assemblage provided six specimens of *F. dimassai*, showing different states of preservation. The best preserved specimen was a subadult (subcode Fd3), measuring 5.1 mm in length, dorsally showing a light scar on the last whorl; the protoconch was intact (Figure 2). Two other specimens (Fd1 and Fd2), measuring respectively 10 mm and 8.5 mm, showed clear damages by predation in the upper part of the last whorl. The net hole recognizable in Fd2 was clearly due to gastropod predation; the same cannot be asserted for Fd1, which showed a wider, most irregular hole. The protoconch was intact in Fd3, but severely damaged in Fd1. The other three specimens consisted in large fragments, each of them including well preserved protoconch and almost three spiral whorls of teleoconch. The specimen Fd4 maintained a wide portion of columella. Such fragmented shells might indicate predation by durophagous fish.

Fusinus dimassai has been described on specimens from Scilla, at the northern entrance of the Messina Strait (Buzzurro & Russo, 2007). Some indications about records from other localities, as Lampedusa, have been never verified (Russo, 2019). The present record of *F. dimassai* from Ustica can be thus considered the first ascertained evidence of a wider distribution than type locality only.

The specimens from the type locality have been all collected in bioclastic sediments from coralligenous habitat, 40-45 m depth. Such original indication disagrees with the attribution, in the same paper, of *F. dimassai* to the Infralittoral zone. It is coherent, by contrast, with the known occurrence of both *Paramuricea clavata* populations and kelp beds, whose association in the Scilla corralligenous habitat has been formerly described by Giacobbe & Spanò (2006).

The strict affinities linking the Scilla seafloors with the “Apollo” bank well explain the similarity in the associated fauna, whose composition is however insufficiently known. The present report of *F. dimassai* from Ustica, following the similar case of *Jujubinus errinae* Smriglio, Mariottini & Giacobbe, 2016 (Giacobbe & Renda, 2019), suggests that the scattered distribution of the Mediterranean kelp beds throughout south Tyrrhenian Isles and banks, might support a peculiar associated fauna with a strongly Atlantic imprint. A different aspect of this linkage has been recently described by Smriglio et al. (2018), according which the distribution of the “smooth” *Jujubinus* complex might related to a speciation process driven by the fragmented distribution of kelp beds along the Surface Atlantic Water inflow.

Conclusion

The present record suggest that Fusinus dimassai belongs to a group of species generally unknown outside the locus typicus, the Strait of Messina, but probably having a scattered distribution across the western Mediterranean, since tied to "residual" Atlantic associations. The occurrence of strict affinities between the Strait of Messina hard bottoms and Tyrrhenian semounts as the "Apollo Bank" are thus further supported.

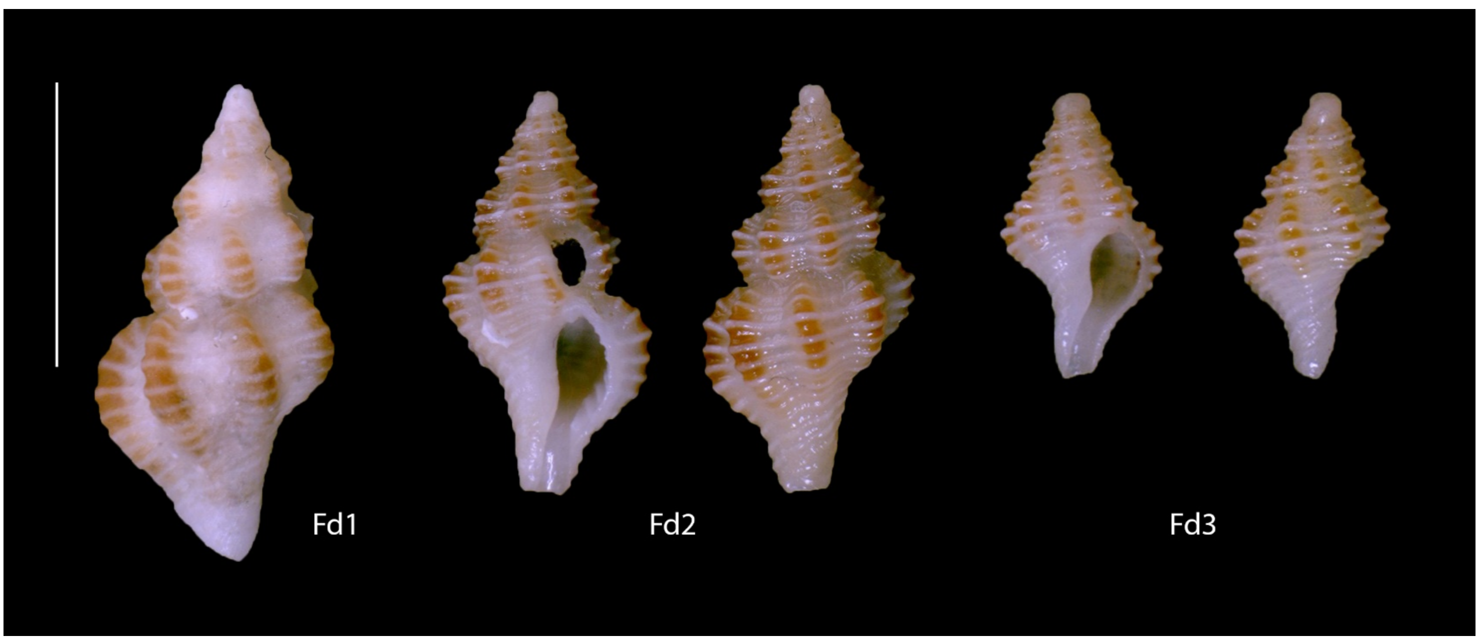

Figure 2. Specimens of *Fusinus dimassai* from Banco Apollo, Ustica (scale bar 5 mm).

Compliance with Ethical Standard

Conflict of interests: The authors declare that for this article they have no actual, potential or perceived conflict of interests.

Ethics committee approval: There is no need ethics committee approval.

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Strait of Istanbul, major accidents and abolishment of left-hand side navigation

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ABSTRACT

Strait of Istanbul is one of the most difficult waterways in the world. It's curved structure, sharp bends which require 12 major course alterations form a unique marine environment for passing vessels. Currents also form a natural difficulty for navigation, especially at areas at the North of Kandilli point. There are three types of currents prevail in the Strait of Istanbul: the first is the surface currents, which finds it's dynamics at the difference of sea levels between Sea of Marmara and Black Sea. Due to this difference in levels, which is around 40 centimeters on average and fed by the rivers flowing into Black Sea, waters run down to Sea of Marmara and to outer seas. Second, is the counter currents and eddies inside the bays. Third is the subsurface currents, formed by the difference of salinity between Marmara and Black Sea, starts from 10 meters to 40 deep and in counter direction with the surface currents. To make this currents system more complicated, in the case of strong SW winds when lasted more than 2-3 days, the whole currents system changes when the surface currents reversed by the wind and above-mentioned difference in sea levels exchange positions to the favor of Sea of Marmara. Due to this unique system of currents, Strait of Istanbul used to have a unique navigational system, too. Starting from ancient times, ships navigating in the Strait of Istanbul used to navigate on the Port side, especially when navigating from Sea of Marmara to Black Sea direction. Thus, ships could get better protection against current. This practice was inked on the paper in 1933 and by the Istanbul Port Regulation through which it became compulsory for ships navigating in the Strait of Istanbul. But as the international regulations on the preventing collision at sea emerged and it appeared that right-hand navigation was established for narrow channels and altering the course to Starboard in the head-on situations has become a rule, the Left-hand side navigation in the Strait of Istanbul started to lead to confusion. There have been three major accidents in the Strait of Istanbul which could be attributed to confusion created by this national rule against the international rule; in 1960, 1966 and 1979, finally Left-hand side navigation in the Strait of Istanbul was abolished in 1982. This article is about these long-term proceedings of Left-hand side navigation in the Strait of Istanbul, concentrating on and analysing three major accidents.

Keywords: Strait of Istanbul, Left-hand side navigation, Thalweg line, Turkish Straits, Collision Regulations, Maritime safety

Introduction

It has been said that a wise man learns from his own experience but a wiser man learns from the experience of others. However, in maritime field, all roads lead to Rome. Every single accident affects all others in the maritime domain. Resulting cost is high and losses are often indispensable. Maritime sector has learnt a lot from the accidents and, during a century, was used to be reactive. It took too long to change this approach. Therefore, the concept of proactive approach with regard to maritime accidents is comparatively new. Preventive measures used to be taken after the accidents, as it was the case with Titanic, Torrey Canyon, Amoco Cadiz accidents and a number of others. Towards the end of 1990's, especially after the Erika (1999) and Prestige (2002) accidents, a proactive/preventive approach started to emerge. Because it was clearly understood, that waiting for an accident to happen to take the lessons costed the indispensable loss of human life as well as vast amount of natural and environmental resources.

With that background, looking at the Strait of Istanbul, we see both reactive and proactive periods with regard to safety measures that has been taken. Proactive period begins after 2000's especially when one-way traffic has been established in 2006 in the absence of a major accident. Reactive period began with the Independenta accident (1979) and continued with Nassia-Shipbroker accident (1994) the first resulted in the establishment of right-hand navigation in accordance with COLREG Rule 9 and te latter resulted in the entry into force of Turkish Straits Maritime Traffic Regulations. The subject of this article is three major accidents that took place including the Independenta accident, which ranked the 9^{th} in the world with regard to amount of oil spilt, and the maritime safety measures took effect following this accident. A common ground was found between these three accidents, which was, a conflict between national and international rules, in which, national rules ordered to alter the course to port in head-on confrontations and international rules ordered the contrary. This article aims to analyze these accidents and set forward whether this conflict played a role in the collisions.

Materials and Methods

Related accidents and maritime safety, field studies, past maritime pilotage experience of the researcher set the groundwork for this study. Three major accidents occurred in the Strait of Istanbul has been analysed to find out their relation with the left-hand side navigation which was in use in pre-1982 era. The ships involved in these accidents has been applied with the same criteria in order to find out the root causes of the accidents. ISailor Electronic Navigational Charts were used in order to obtain nautical data and positions. The accidents have been approached by taking the human element into account as a primary figure and due to this approach and in order to create a perception of empathy, a story-telling style presided in the wording. In analysing the three major accidents, a controversial common ground was found; which was the head-on situation at the encounter before each accident. The actions of encountering vessels were analysed and findings were left for discussion.

Reactive and Proactive Approaches in Maritime Safety

General maritime law was first codified in 2000 B.C within the Hammurabi Code and at that time, accidents were accepted as the act of gods (İstikbal, 2012). Towards the end of the 19th century, conversation took place between the main sea-related nations, such as Great Britain and France, especially to establish common rules for accident avoidance in the English Channel (Soltani, 2009). The first maritime measures regarding the maritime safety took place after the *Titanic* accident, 1912, after which the first international conference convened and led to the first International Maritime Convention, SOLAS 1914, and enforced in 1919. Another example was the M/T Torrey Canyon accident, in 1967, a tanker, which ran aground, while entering the English Channel and spilled her entire cargo of 120,000 tons of crude oil into the sea. This was the biggest oil pollution incident ever recorded up to that time (IMO, 2019). And, in consequence, IMO Legal Committee was created in 1967, CLC Convention and International Convention related to the Intervention on the High Seas in Cases of Oil Pollution Casualties were concluded in 1969 followed by Fund Convention in 1971 and MARPOL Convention in 1973. M/T Exxon Valdez case was another major accident that was led to legislative measures. The tanker went aground in the Prince William Sound in Alaska in 1989, caused the spill of the 37,000 tons of crude oil -almost one fifth of her whole cargo, which was 162.000 tons. This was the largest oil spill in US waters until that date. Subsequently, US Oil Pollution Act was enforced in August 1990, MARPOL amendments were made in March 1992 which brought double hull standard for new building tankers and a phase out period for the existing single hull tankers. Erika and Prestige tanker disasters, in 1998 and 2002 respectively, which were caused to 20.000 tons and 63.000 tons of oil spilt into Atlantic Ocean off Spanish and French coasts, alerted the European Union to take specific measures. Resulting actions were the ERIKA 1 and ERIKA 2 packages in March & December 2000, Phase out of single-hull tankers, creation of European Maritime Safety Agency (EMSA), reinforcement of Port State Control system (PSCs), adoption of FUND II in 2003, preparing of EU ERIKA 3 Package, in December 2008, set up ship-owners and Flag States obligation in the event of oil pollution.

All examples given above indicate that the approach of maritime sector with regard to maritime safety during these periods was reactive. However, with the beginning of the 21st century, a new approach has emerged led by the International Maritime Organization. Instead of waiting for accidents to happen and take steps afterwards, IMO decided to be proactive and started to review its framework accordingly. In 2002, IMO adopted the "Guidelines for Formal Safety Assessment (FSA) for use in the IMO rule-making process". FSA was, as described by IMO, *"A process used for ensuring that action is taken before a disaster occurs"*. It is a structured and systematic methodology, aimed at enhancing maritime safety, including protection of life, health, the marine environment and property, by using risk analysis and cost benefit assessment. A new era has begun, and member countries were encouraged to report all near misses and incidents in order to assist to bringing-to-life of this new approach.

Left-Hand Side Navigation in the Strait of Istanbul

Currents

The Strait of Istanbul has unique characteristics. Located between the Black Sea and the Sea of Marmara, it is a waterway 32 kilometers in length and with an average width of 1.5 kilometers. The length of its coasts measures 55 km on the European side and 35 km on the Asian side (İstikbal, 2001). From this, we can see that the European coast is 36% longer and comparatively more indented. This is also one of the indirect factors for currents being more effective on the Asian side.

The axis passing from the mid-points at both ends of the Strait forms an angle of 25 degrees from Geographical North. The air route over the strait is 025 degrees towards the Black Sea and 205 degrees towards the Sea of Marmara. These routes are also the average of total passing routes of ships in the Strait. If we convert this to wind direction, it is the NE-SW axis-which are the prevailing winds in the Strait. This is a factor for accelerating the southerly currents and The extra supply of water in the Black Sea by rivers produces a difference in the sea level between the northern and southern ends of the Strait, due to which a constant current occurs; but in this channel, from its having seven sharp bends, counter-currents and eddies are formed on either shore, as in a river.

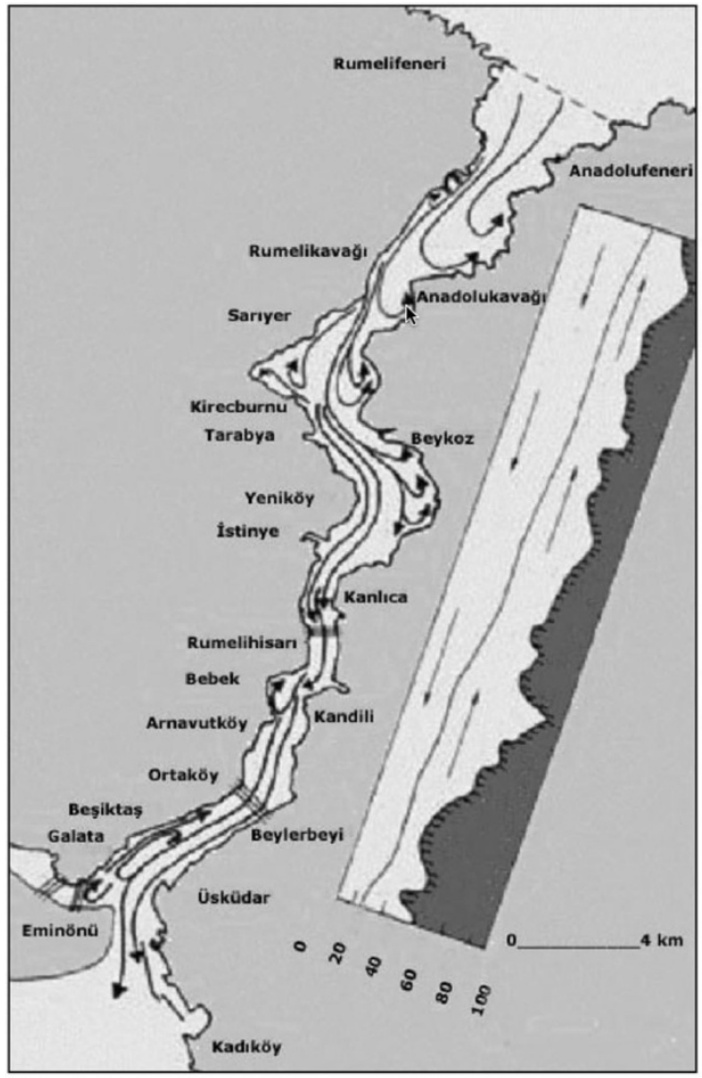


Figure 1. Surface and subsurface currents at the Strait of Istanbul (Seyir Hidrografi ve Oşinografi Dairesi Başkanlığı)

The currents in the Strait of Istanbul have a fast response to sea level differences between the Black Sea and the Mediterranean (Özsoy et al, 2002). Black Sea is about 40 centimeters higher than the Sea of Marmara and 60 centimeters higher than the Aegean. Within the two-layer current system in the Strait of Istanbul, this difference in the water levels at both ends constitute an upper current which has an average speed of 3-4 Knots towards the Sea of Marmara (Figure-1). The lower layer is about a half compared to the upper layer, with regard to velocity and volume of the water transferred (Figure-2).

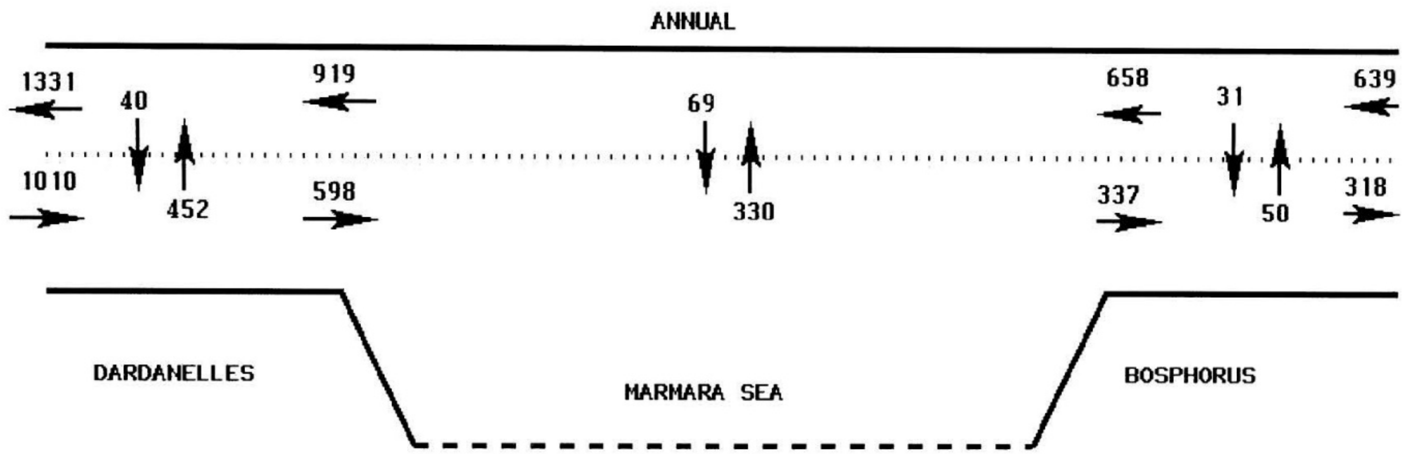


Figure 2. Annual volume fluxes (km^3/year) across the compartments of the Sea of Marmara and straits (Tuğrul et al. 2002).

Contributions of precipitation and river runoff are of nearly equal volume (300 and 320 km^3/yr respectively), totaling a fresh water input to Black Sea which is about twice as large as the evaporation (350 km^3/yr) (Ünlüata et al., 1990). As the only water output to high seas, Black Sea sends this water through the Strait of Istanbul first, via upper layer, which forms a height difference of water on either side. The warm and more saline lower layer, called Mediterranean water, flows towards the Black Sea. These two layers, despite a neutral area they have in between, do interact and this interaction is indicated in the Figure 2. The upper and lower layers constitute the current dynamics of the Istanbul Strait in. particular, and the Turkish Straits system in general. From the aspect of maritime transportation, the lower-layer is negligible with regard to its effect to the passing vessels. Upper layer is what this article interested about. Surface currents, which can increase up to 6-8 knots in speed, are one of the most important handicaps for navigation through the Straits (İstikbal, 2006).

By analyzing Figure 1, we can find out that the currents will be more effective in the Asian side in certain parts of the Strait of Istanbul, because diverting arms such as Tarabya-Yeniköy area and Bebek Bay-Akıntı Burnu area directs the current towards the Asian shores. In fact, Strait of Istanbul can be divided in two main parts: The Southern Part and the Northern Part. Kandilli Point can be appointed as a dividend between these two. From the holistic perspective, it can be said that surface (upper layer) currents are more effective for navigating vessels in the Southern part compared to the Northern part.

The dominant current system is effective most of the year-in the case of prevailing NE winds. But in the Istanbul Strait, SW winds are also effective in certain periods of the year. When SW winds are effective, the current regime changes a lot throughout the Strait. There has been a number of accidents in the existence of strong SW winds and northerly currents (Locally called Orkoz) in the Strait. Petar Zoranic-World Harmony, Nassia-Shipbroker accidents are some dramatic examples.

Left-hand side Navigation in the Strait of Istanbul

Experienced navigators and local pilots, as far as regulations allowed, used to take advantage of the currents in the Strait of Istanbul. When they entered the Strait of Istanbul from Sea of Marmara, North bound, they used to cross towards European shores after having passed abeam Kızkulesi (Maiden's Tower). Let's take a Northbound vessel passing through the Strait as an example. The ship must struggle with the strong current off Haydarpaşa Breakwater first. Experienced captains would approach the starboard side after passing the breakwater lighthouse. Here the current is neutral or more or less northbound. However, strong currents take over the ship again abeam of Maiden's Tower literally pushing the ship from the starboard side. From 1934 until 1982, ships used to cross directly towards Ortaköy from this point. After crossing the Maiden's Tower, they would alter course to the opposite shore. This was because currents which are strong on the Anatolian shores were weak on the European side. In fact, a reverse current close to the coastline at Dolmabahçe and Beşiktaş leans towards Ortaköy and Galatasaray Island up to the Akıntı Burnu, or Hermaion Point as it is known in mythology. Following this route can save up to 45 minutes in a Strait passage. That was probably how Left-hand side navigation emerged in the Strait of Istanbul. Officially remained in force between 1934-1982, left hand navigation used to b e known as the-most convenient method of passage for ships in order to minimize the effect of counter currents. Figure-3 indicates the Left-hand side side passage system that remained in force between 1934 and 1982. There were 2 areas where ships crossed from one shore to the other. For north-bound passage, the first crossing was starting in front of Kızkulesi (Maiden's Tower) ending at Ortaköy area where Left-hand side navigation should have fully confirmed, and other was starting in front of Tarabya bay ending at Umuryeri Banks, where return to right-hand navigation took place. For South bound passage, the same crossings were held in vice versa.

So far, it has been emphasized that Left-hand side navigation was very convenient with natural and morphological conditions of the Strait, especially upper currents. One question may arise: was it voluntary for passing ships to take this advantage or were there any rules making this system compulsory to comply with? The answer is the latter. The regulations regarding the Left-hand side navigation were as follows in chronological order:

1. **Istanbul Port Regulation (07 August 1933):** According to this regulation, in contrast with right-hand side navigation, which was in force in most Straits of the world, Left-hand side side was established in the Strait of Istanbul (Oğuzülgen, 2016). New Istanbul Port Regulation drafted to replace the Istanbul Port Ordinance and this new Regulation was was published on the Official Gazzette no. 2471 in 25 July 1933 and entered into force. According to Article 62 of this new Regulation, effective date of Article 17, which was regarding to Left-hand side Traffic, would be 6 months later. The mentioned ***Article 17*** of 1933 Regulation was as follows:

"Article 17: *Whenever possible and safe from the danger, all ships crossing the Black Sea Strait towards the Black Sea will navigate on the the Port side of the Bosphorus, which means Rumeli side, and the the ships from the Black Sea, will navigate on the starboard, which means on the Anatolian side. Article 25 of the Regulations for Preventing Collision at Sea shall not be applied on the Bosphorus. Şirketi Hayriye ships will navigate according to the berths where they will stop by."*

This new regulation, which established Left-hand side navigation in the entire Strait, entered into force on 25 February 1934.

2. **Decision of the Council of Ministers (11 October 1934**): Implementation of Left-hand side in the entire Strait formed a full contradiction with the *Regulations for Preventing Collision at Sea*, due to which, the Council of Ministers revisited and amended the Article 17 of Istanbul Port Regulation in 1934. According to the amended Article 17, the former Article 17 (above) remained the same as Paragraph 1 and the following Paragraph was added as Paragraph 2:

"Article 17: *The application area of this article is between the line connecting Kızkulesi and Ortaköy Mosque in the South and the line connecting Kireçburnu Lighthouse to Umuryeri Bank Lighthouse in the North. Apart from this field, the Regulations for Preventing Collision at Sea are applied. The ferries to visit the piers on the shores of this area navigate according to the piers to be visited."*

According to 1934 amendment, the Left-hand side navigation in the Strait of Istanbul was limited to a certain area, namely between Kızkulesi and Ortaköy Mosque in the South and Kireçburnu Lighthouse to Umuryeri Bank Lighthouse in the North (Oğuzülgen, 2016). In the rest of the Strait, the appropriate rules of the Preventing Collision of the Sea for right hand navigation in a channel were to be applied. The coverage area of Left-hand side navigation consisted only a few miles less than the half of the total length of the Strait (7 out of 18 Miles).

3. **Istanbul Port Regulation (25 December 1965)** : The 1934 Port Regulation needed an update after 21 years of experience. With a new regulation which was announced by the Decision of Council of Ministers on 19 November 1965 and entered into force by publishing on the Official Gazette No. 12186 on 25 December 1965, some new measures were introduced as well as some articles of the previous Regulations were amended. As for the new measures, the speed limit for passing ships was set to 10 knots (Art. 29), fishing was prohibited (Art. 44 & 45) the local traffic ordered not to impede and keep out the way of passing non-stopover traffic through the Strait (Art. 27) and a change was made on the application limits of Left-hand side traffic system (Article 26). The related article of the new regulations wea as follows:

"Article 26: *Ships passing towards North through the Strait of Istanbul will proceed on the Left-hand side of the thalweg (median) line in the Strait (European Side) and South-bound ship passing towards Marmara direction will proceed on the Left-hand side (Asian side) of the thalweg line. This rule will be applied between the line from Kızkulesi to Ortaköy*

Mosque in the South and the line between Tarabya Point to Umuryeri Bank in the North."

The Article 26 of 1966 Regulation had no big difference from the Article 17 of 1934 regulation; the Kireçburnu Point in the 1934 regulation was changed to Tarabya Point, which was only 3 cables in the NW. So we can assume that the Left-hand side navigation was kept as it was in the 1965 Regulation.

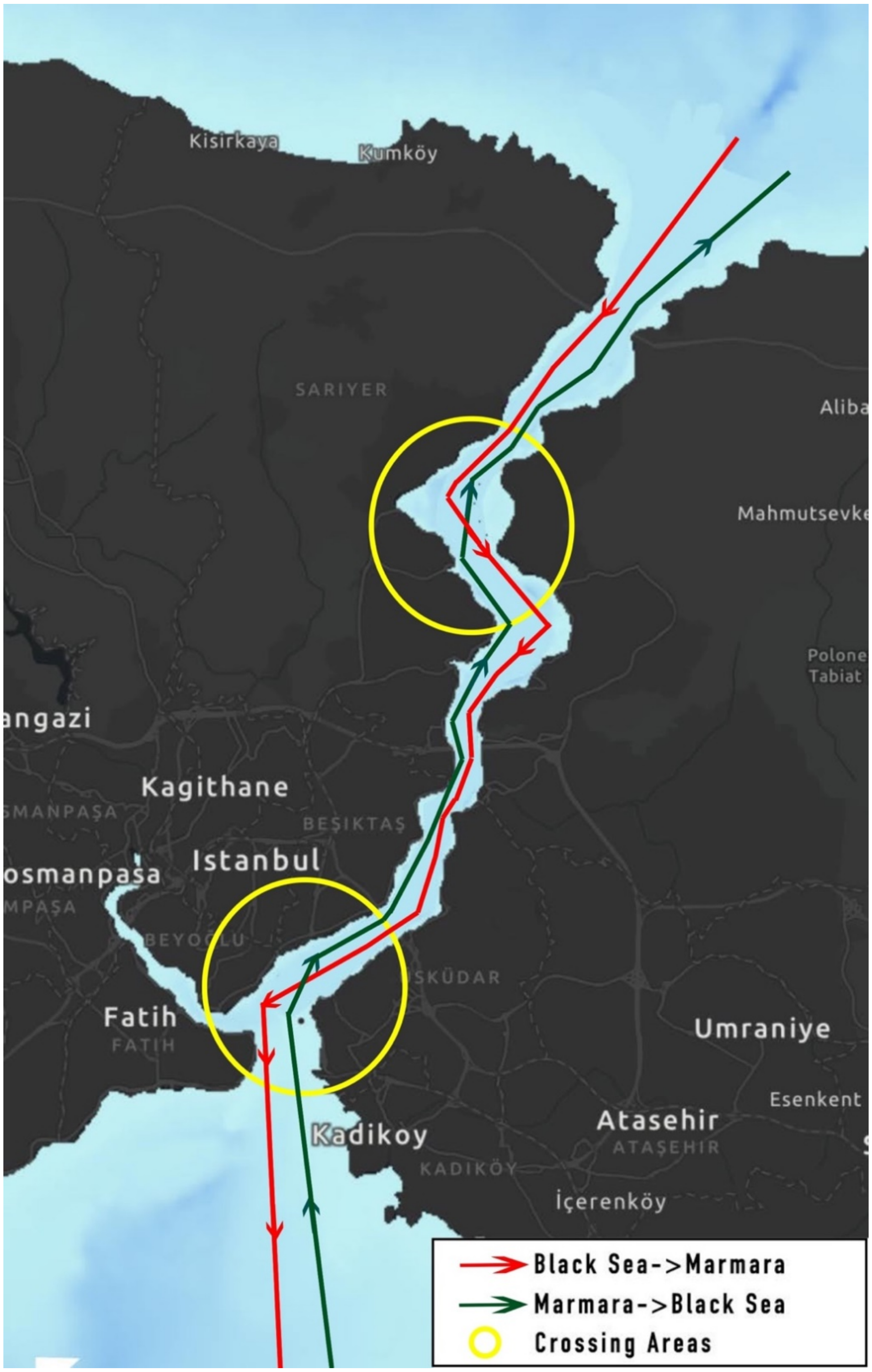


Figure 3. Left-Hand side navigation system in the strait of Istanbul, which remained in force between 1934-1982

Accidents in Strait of Istanbul: From Inactivity to Reactive and Proactive Periods

Several maritime accidents happened in the Strait of Istanbul before the most extensive set of regulations put into effect in 1994. Tragic accidents began in 1960 with Peter Zoranic-World Harmony collision after which no action was taken and continued with M/T Lutsk-M/V Cransky Oktiabr collision in 1966, after which no action was taken either. The latter was obviously due to Left-hand side navigation that was in effect in the Strait and that was pointed out in the statement of Captains after the accident. However, termination of this application (Left-hand side navigation) took 16 more years and finally COLREG Rule 9 for narrow straits was implemented in 1982.

The maritime accidents and their subsequent reflections in the maritime safety measures are shown in Table 1.

M/T Peter Zoranic- M/T World Harmony Collision

a. **Accident:** On August 27, 1960, the shipyard "III Maj" delivered the tanker "Petar Zoranic" to Yugoslav Tanker Ship, the Government company of Yugoslavia. It was named after the Croatian writer Petar Zoranic, author of the first Croatian novel, *The Mountain* (Kvaternik, 2018). It was launched on September 20, 1959. Her IMO number was 5613855, GT 17.830, and she was carrying 25,400 tonnes. He was enlisted in the fleet of the Zadar shipping company "Jugotanker", today's "Tankerska plovidba", on September 27, 1960. The tanker "Petar Zoranic" was the largest ship built in Yugoslavian shipyards until 1960.

Table 1. Major Accidents in the strait of Istanbul and counter measures

Maritime Accidents in The Strait of Istanbul And Subsequent Safety Measures					
Accident	**Type&Date**	**Location**	**Possible Cause**	**Result**	**Resulting Action**
M/T Petar Zoranic M/T World Harmony	Collision 14/12/1960	Kanlıca	Left Hand Navigation	52 Lives Lost 22.000 Tons of Oil Spilt	No measures were taken
M/T Lutsk M/V Cransky Oktiabr	Collision 01/03/1966	Maiden's Tower	Left Hand Navigation	12.000 Tons of Oil Spilt	No measures were taken
M/V Evriali M/T Independenta	Collision 15/09/1979	Near Haydarpaşa Breakwater	Left Hand Navigation/ Pilot Embarkation/Disembarkation Locations	90.000 Tons of oil spilt & burned	Left hand navigation terminated-COLREG Rule 9 for narrow channels established.
M/V Shipbroker M/T Nassia	13/03/1994	Northern Entrance/In front of Kavak Point	Not suspension of traffic/Pilot Embarkation-Disembarkation Locations	9.000 Tons of crude oil spilt& 20.000 tons of Crude Oil burnt	Suspension of traffic during passage of large tankers/Turkish Straits Maritime Traffic Regulations/IMO approved COLREG Rule 10 implementation

Milestone maritime accidents in the Strait of Istanbul and subsequent actions taken

Table 2. MT Petar Zoranic/MT World Harmony ship characteristics

	M/T Petar Zoranic (South-Bound)	M/T World Harmony (North-Bound)
Date-Place Built	1960-Yugoslavia (BRODOGRADILISTE III MAJ, Rjeka, Croatia)	1954-UK (Vickers-Armstrong Ltd., Barrow-In-Furness)
Owner	Jugoslavenska tankerska plovidba (Jugotanker)	Stavros Niarchos Ltd., London
Type &Flag	Tanker-Yugoslavian	Tanker-Greek
Pilot Onboard	Yes- Capt. Cevdet Çubukçu	No
Cargo	Fully Laden Gasoline: 12.000 T & Diesel Oil: 11.330 T	No Cargo-Dirty Ballast
DIMENSIONS (Lxbxd)	192.4x25x9.8 m	202.1 x 26.4 x 10.5 m
Tonnage	17830 GT	20992 GT
Max.Speed	17.5 Knots	15.5 Knots
Engine & Propeller	Steam Turbine, Single Screw	Steam Turbine, Single Screw

She ended its voyage forever in a horrific fire on December 14, 1960, the 108th day of navigation. On December 13, 1960, "Petar Zoranic" departed from the Black Sea port of Tuapse, loaded the cargo into the front tanks of 12,000 tons of gasoline and into the stern tanks of 11,330 tons of diesel fuel. The tanker was under command of Capt. Anton Sablic from Kostrena. It was about 01:00 O'Clock, just starting of the day of 14th December, 1960, when tanker Petar Zoranic arrived at the Northern entrance of the Strait of Istanbul, where she picked up a local pilot. Capt. Cevdet Çubukçu was the pilot who climbed up the ladder extended from the tanker. Born on 1 October 1926 and started his maritime business after secondary school Capt. Cevdet Çubukçu was just 34 years old at that time. Having worked on vessels named Kaptan Uzunoğlu and Hatay owned by DB Cargo Lines, he had begun his pilotage career in 1955. He was almost at the beginning of his pilotage career when he got onboard M/T Petar Zoranic. And it was an extraordinary day in the Strait. Because strong Southern winds were prevailing in Istanbul since a couple of days which provoked a reverse current in the Strait that reached to 5 knots under the effect of continuous winds which exchanged the sea level difference on advantage of Marmara coast and higher waters of Marmara started to run towards the lower level waters of Black Sea with wind helping by pushing the surface waters. This was the reverse current which locally called as “Orkoz”. Orkoz always created a different current regime in the Strait, making the navigation more difficult. And, in 1994 Regulations, traffic started to be suspended when Orkoz currents exceeded 4 knots of speed. At that day, it was 5 knots, but unfortunately, at that time period, there was no regulation existed to suspend the traffic in the Strait. So, tanker Petar Zoranic entered the Strait, picked up the pilot at Kavak point, upon arriving on the bridge, Capt. Cevdet Çubukçu and Capt. Anton Sablic shaked hands, conning of the ship -most probably- delivered to pilot and passage through the Strait commenced. Soon after, the tanker arrived to Köybaşı point, which required the sharpest turn-about 80 degrees- in the Strait, and after the turn completed, started to move towards Kanlıca point on the Asian shores as she should follow the Left-hand side side of the Strait due to Port Regulations. At that time, a marginally larger tanker, M/T World Harmony, was proceeding to North. She was under ballast, without cargo, but her tanks were not gas free which meant they were prone to an explosion.

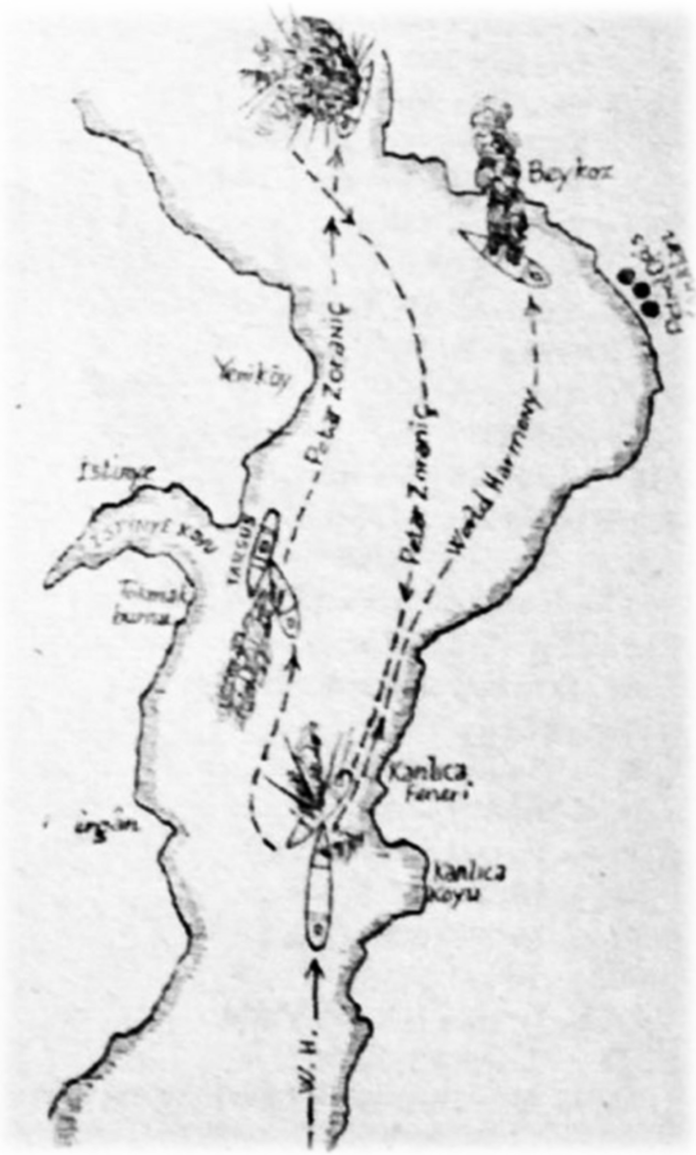


Figure 2. Petar Zoranic- World Harmony accident on an estimated simulative diagram (Koçu,1963)

On the bridge of tanker World Harmony, Master Capt. Aristides Bardzis, was commanding. Captain Aristides Bardzis was born in 1913, in Psara, a small island in the Northern Aegean. After graduated from Syros Technical School, he started to work onboard ships at young age, and, he became a deck officer at the age of 24. After age 32, he started to work with Niarchos company, owned by Greek shipping tycoon Stavros Niarchos. Captain Bardzis started working onboard of World Harmony in 1957 and first two years, the tanker navigated to and from Persian Gulf to European markets and in 1959 started navigation to Black Sea destinations. So, he had more or less one year experience in the Strait of Istanbul, with a number of passages, about which, the Author is of the opinion that would be insufficient to be familiar with the shape of the Strait. Having passed through the narrowest part of the Strait, the Kandilli area, Capt. Bardzis proceeded further, and he noticed the anchored passenger vessel S/S Tarsus off Tokmak Burnu near İstinye Bay, where at that time a large shipyard existed, the İstinye Shipyard. Aligned ship's heading to SW-NE direction with the effect of wind and current, S/S Tarsus was almost head-on situation with the World Harmony. And Capt. Bardzis also noticed the south-bound tanker Petar Zoranic, just took the sharpest bend around and proceeding. It was about 02:30 after midnight. Two large tankers moved towards each other. Head-on situation. Pilot onboard Petar Zoranic knew that according to regulation he should be closer to Asian side. So, altered the course more to port. At that time, due to head-on situation and probably in confusion with the Left-hand side navigation rule, Capt. Bardzis was proceeding on the Asian side as well. His vessel was expected to proceed on the European side (port side of the channel), according to the rule, but was proceeding on the Asian side, (Starboard side of the channel). On the opposite side, Peter Zoranic sent out emergency whistle signals but nothing helped, and at the very last moment, to avoid the accident, Peter Zoranic altered “hard to starboard”, but it was too late... In about 02:40, bow of the World Harmony crashed into the port bow of "Petar Zoranic" at an angle of approximately 45 degrees in the tank area 2, which contained gasoline. A terrible explosion ensued; gasoline flames covered the front superstructure. Other tanks containing gasoline exploded one after the other due to the force of the impact. There were also explosions on the World Harmony. She had no cargo, but from the previous trip, the tanks were left uncleaned so that an explosion occurred in them. Both tankers were burst into flames; fuel from Petar Zoranic was spilt onto water and caught fire. Although the coast was relatively close, 150 meters away, it was not safe to swim to shore as pouring fuel from Petar Zoranic turned the Strait into a river of fire, with the strong current running towards Tokmak Burnu area. Flames rose tens of meters up, and a fire on the water threatened shores on the coast. Petar Zoranic and World Harmony, after the first crash, left without command and while World Harmony drifted towards İncirköy Banks area near Beykoz and stranded there, Petar Zoranic, with the rudder remained hard to starboard and effect of SW winds, drifted towards Tokmak Burnu area near İstinye, where Turkish passenger ship “S/S Tarsus” was at anchor, a 140-meters long luxury passenger vessel prepared for an exclusive cruise to United States. Petar Zoranic drifted towards and allided with S/S Tarsus, which prevented her to crash into shore, but, also set the ill-fated passenger vessel in fire.

Petar Zoranic stayed there allided with S/S Tarsus for 10 minutes, and then, started to re-drift with the effect of wind and current, towards the Beykoz area, to where World Harmony got stranded, and drifted further northwards, and could stop drifting near Selvi Burnu area, which is as far as 2,5 Nautical Miles from the place where the collision took place. Intervention by firefighters and soldiers saved dozens of homes on the coast in Beykoz Bay. In the shallows of Selvi Burnu, shaken by occasional explosions, "Petar Zoranic" continued to be ablaze for the next 55 days. It was ablaze until all fuel on board had been burned, until February 6, 1960. A total of 53 lives were lost in this tragic collision and three ships were destroyed. Capt. Cevdet Çubukçu, pilot onboard Petar Zoranic, lost his life at the very first seconds of accident. Masters of both ships were also amongst the victims. 21 Croatian sailors, 29 Greeks, two Turkish customs officers and a Turkish pilot lost their lives. Two Turkish customs officers' onboard S/S Tarsus. It is the largest maritime accident in Croatian history and one of the greatest tragedies of its kind in the world.

Figure 3. Anton Sablic, Captain of tanker Petar Zoranic

Figure 4. The master aristides Bardzis (Nautical History, 2010)

Figure 5. Captain Cevdet Çubukçu, pilot onboard tanker Petar Zoranic

b. **After-accident era:** The maritime accident was investigated by a fourteen-member commission made up of experts on maritime law and casualties, as well as experts in maritime insurance from Turkey and the former Yugoslavia. In the voting for assessing the responsibilities of ships involved in accident, thirteen members voted for World Harmony to be at fault for 97 percent and the tanker Petar Zoranic only 3 percent. However, one committee member voted that 87 percent were guilty of tanker Zoranic and 13 percent of World Harmony.

After the accident, some safety measures were taken in the Strait. According to daily Cumhuriyet newspaper published on 20/11/2019, these measures were:

1. Night-time passage of laden or ballast tankers of all sizes and ships carrying dangerous cargo prohibited,
2. Speed limit was set to 10 Knots as maximum,
3. Distance between passing ships wea set to 1000 yards as minimum (914 meters)

However, these measures mentioned in the newspaper were not enforced by law and not known if applied at all, even if they did, not for long and were not remained as permanent, faded away as the time went by after the accident.

c. **Accident analysis:** In the light of information above; and according to a number of criteria, the following conclusions were made:

   - **Navigational Errors:** While Yugoslavian tanker Petar Zoranic followed the appropriate course in compliance with Istanbul Port Regulation, as set forward at Figure 3, the Greek tanker, World Harmony was on the right-hand side of the channel, which meant she followed the wrong course, not in compliance with above-mentioned Regulation, which was in force at that time. Compliance: Petar Zoranic.

- **Pilot onboard:** Having a local pilot onboard, the Yugoslavian tanker, M/T Petar Zoranic had fulfilled the necessary initial requirements for a safe passage. Compliance: Petar Zoranic.
- **Meteorological/morphological difficulties**: As another factor, Southbound M/T Petar Zoranic was proceeding against the wind and current, at the time of accident strong SW winds and northerly current were prevailing, as local people call it, the "orkoz". That possibly made the navigation more difficult for both vessels, especially for the northbound one.
- **Speed in the strait:** Another issue was the speed of both vessels. According to estimation, Southbound and laden Petar Zoranic was not exceeding 10 knots over the ground, as she proceeded against the wind and current. But, the speed of World Harmony could be around 15 knots-and even more. Petar Zoranic has the points here.
- **Left-hand side navigation and crossing areas**: The most controversial issue with regard to this accident is the "left hand navigation" that was in effect in the Strait of Istanbul during that time. It was a long-established rule, however, effective since 1933, but as the right-hand navigation was -also- partly used in the Strait, it can be claimed that this two different and in fact, opposite passage system led the captain of World Harmony into confusion. Two vessels in reciprocal courses used different rules: World Harmony complied with the international rule (Rule 14 of the collision regulations) which ordered to alter the course to starboard in head-on situations while the Petar Zoranic complied with the local rule (Article 26 of the Istanbul Port Regulation) which ordered to follow the Port-hand side of the channel (That included, by the essence, to alter the course to port for vessels in reciprocal courses). But, however, this was no excuse for the Captain of World Harmony. The long-established customary rules in the maritime transportation ordered the captain to be "prudent and completed". That meant for the Captain of World Harmony to have the proper local knowledge including the local rules or to have a local pilot picked up at the Southern entrance of the Strait.

M/T Lutsk-M/V Cransky Oktiabr Collision

a. **Accident:** Strait of Istanbul saw one of the most terrific accidents on 1st of March, 1966, in which two ships, one being a bulk carrier the other a tanker, of Black Sea Shipping Company, largest shipping company of the world during that time, collided. Black Sea Shipping Company was a Ukrainian shipping company based in Odessa. During Soviet rule, the company held the title of world's largest shipping company for several years and was instrumental in important foreign trade and international aid initiatives of the Soviet government. As a result of strong political and economic relationship and collaboration between Soviet Union and Cuba (Remember October 1962 Cuba Missile Crisis) at that time, Kransky Oktiyabr had her cargo loaded from the port of Puerto Padre of Cuba, 15.500 tons of sugar in bags. On Friday, March 1, in the afternoon, Kransky Oktiyabr passed through the Strait of Çanakkale and by night, approached the Strait of Istanbul. Loran Antonovich Kozyr, Captain of Kransky Oktiyabr, felt confident on the commanding bridge, when the Chief Officer asked if he required a pilot for passage, he answered twice: "No, let's go without a pilot". The moon was shining, a clean night, visibility was good, and state of sea was calm. At 22.07, they passed the lighthouse of Ahırkapı and at 22.15, they started turning to the Starboard at 048° around the Kızkulesi lighthouse. Ahead was the most responsible section - the line of transition to Left-hand side traffic and the road close to European side in front of Dolmabahçe Palace extending to Ortaköy area, on which ships often stayed on anchor, whose had weak anchor lights which were poorly visible at night.

Figure 6. Positions 4 minutes before and place of collision of Lutsk and Kransky Oktiyabr

Suddenly, it was at 22.18, the third officer standing at the radar reported: "I see a large vessel on the screen of the radar". It was, 1.5 Nautical Miles ahead, red port side light and two white mast lights were visible. This was the tanker "Lutsk". She was proceeding to South-West, in front of Ortaköy area, closer to the European coast- Starboard side of the Strait, on a wrong course. According to the rules of Left-hand side navigation in the Strait of Istanbul, as set out in Figure 3, she was expected to be closer to the Asian coast, not to the European coast. Captain Kozyr ordered the engine to slow down, "Dead Slow Ahead!". On the opposite side, under the command of Captain Vladimir Tolochko, the tanker Lutsk was laden with 32.000 Tons of Crude Oil. Captain Kozyr, on the bridge of Kranskiy Oktiyabr, saw Lutsk on the port side of his vessel, and knowing her being on the wrong course, he decided to pass "red to red" (Port-to-Port). But at the very moment, he saw the Lutsk altering her course to port. He wanted to do the same, and ordered "hard to port". But, the Kransky Oktiyabr, being at the slowest speed, did not answer to the command. Then Captain Kozyr ordered "Hard to Starboard" accompanied by one short signal with the whistle. In response, he heard two short signals from the Lutsk. That meant bad news, she was insisting to alter her course to Port! Rules were rules, it was obvious that Captain Tolochko wanted to go back to the correct course ordered by the Port Regulations. At this point, Captain Kozyr, altered his mind again. "Engine half ahead, rudder hard to port" he ordered, and gave two short signals declaring this decision. Onboard the Tanker Lutsk, Captain Tolochko interpreted these maneuverings of Kransky Oktiyabr as if they were done to avoid collision with a ferry crossing from Asian side to European side (There was such one indeed, Capt. Tolochko had said about this interpretation of him to Captain Kozyr after the accident). But the vessels were very close now, only a few cables. The distance between the vessels was about two miles when they first saw each other, and now, it was less than a mile. Captain Kozyr noticed that it would not be possible to avoid collision with rudder and engine commands. The best way was to stop the movement of the ship. Then he ordered "Full astern and let go the starboard anchor". At 22.22, tanker Lutsk crashed onto Kransky Oktiyabr. Contact point was on the starboard side, just ahead of the accommodation of the Lutsk and on the stem of the Kransky Oktiyabr. The stem of Kransky Oktiyabr punctured a hole on the shell of the tanker about 5 meters in diameter through which crude oil started to spill immediately. With the effect of the collision, similar to the push of a tugboat, tanker Lutsk pushed and turned the bow of Kransky Oktiyabr towards her starboard side and both vessels now turned to the Sea of Marmara and proceeded further, with Lutsk continuously leaking crude oil. Some 750 tons of crude oil was spilt to the sea until both vessels arrived to Sea of Marmara, where two ships were separated from each other and Tanker Lutsk proceeded further South in order to flush the remaining of the punctured tank and Kransky Oktiyabr proceeded to the Ahırkapı Anchorage. It was nothing but only a luck that the crude oil did not catch fire during the crash and the tank did not explode, most probably the fullness of the tank prevented gas accumulation over the surface which if not, could cause a fire.

But, this did not mean that the crude oil that was spilt would never catch fire. In fact, that happened after one and a half hours from the accident, at Karaköy district, where local ferries from and to Kadıköy area were coming alongside on a pantoon connected ashore (See figure 12). One of these local ferries, named Kadıköy, built in 1912 in France and 54 years old at that time, was moored to the pantoon at the time of accident, and would leave for Kadıköy (district) at 23:45, but due to an intense smell of crude oil in the air, the ferry which already embarked the passengers, was kept waiting. It was around midnight, due to unknown reason, perhaps, according to some eyewitnesses due to an overthrown aflame piece of paper from the front section of the ferry, caused a spreading fire over the sea surface. The passengers of the ferry, which were around 1500, abandoned the ferry immediately. The Ferry was on fire and fire broke the shore connections -ropes- and set the ferry adrift without being under command. Ferry drifted towards Sirkeci area after which rescue vessels belonging to Turkish Naval Forces Command took her under control.

The ferry "Kadıköy" was totally destroyed by the fire. The fire spreaded over the sea further and the front section of Italian-flagged cargo/passenger vessel San Georgio, which was alongside at Karaköy berth a few hundred meters away, also caught fire. The ship was unmoored and moved to anchorage. The other ships at the nearby berths, Turkish cargo/passenger vessels M/V Sus and M/V Ege were also taken to anchorage as a precaution. Galata bridge was also suffered from fire, the shops under the bridge were damaged from fire. Across the pantoon, all the seafront window glasses of Rıhtım Branch of Ziraat Banks were melted. This was the second incident at which oil and derivatives caught fire on the water surface of the Strait of Istanbul after an accident. The first one was Petar Zoranic-World Harmony accident.

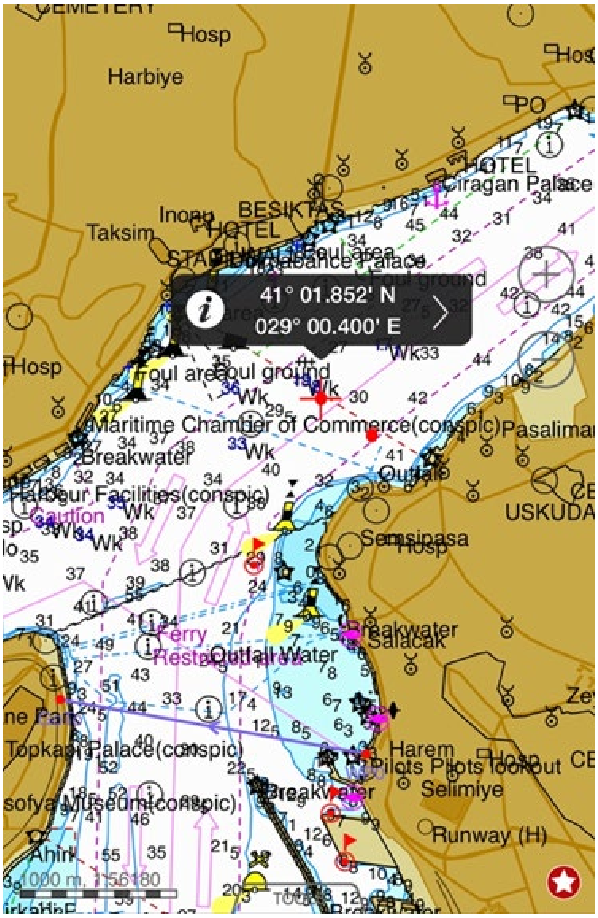


Figure 7. Exact position of the Lutsk-Kranskiy Oktiyabr accident. Position fixed by the officers on the bridge of Kranskiy Oktiyabr: 41 01.85 N and 029 00.40 E

Figure 8. This photo, Property of Özdemir Gürsoy and published on Daily Milliyet Newspaper on 03.03.1966, shows the damage on the starboard side of tanker Lutsk.

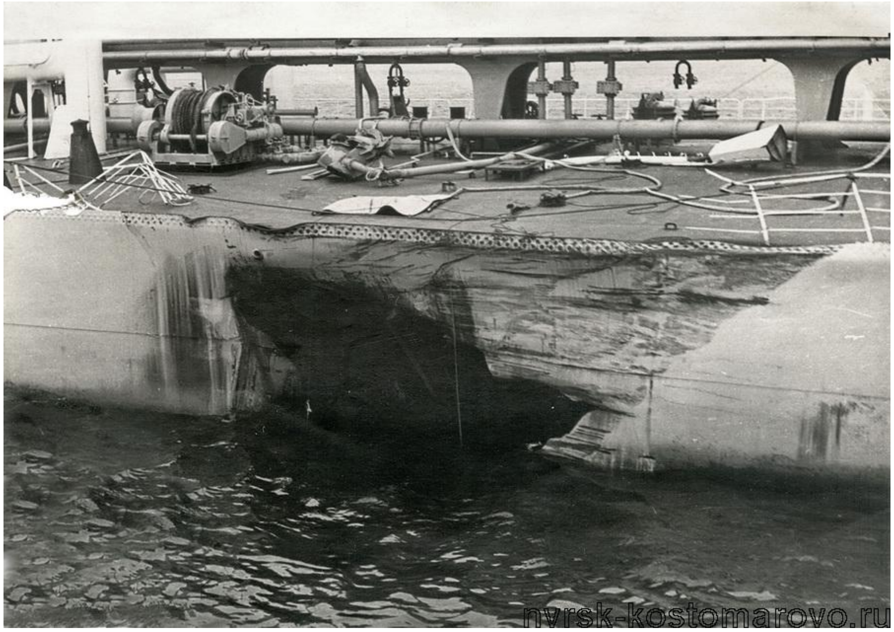


Figure 9. The damage on the single-hull Lutsk shows the gravity of possible disaster which was very near to happen.

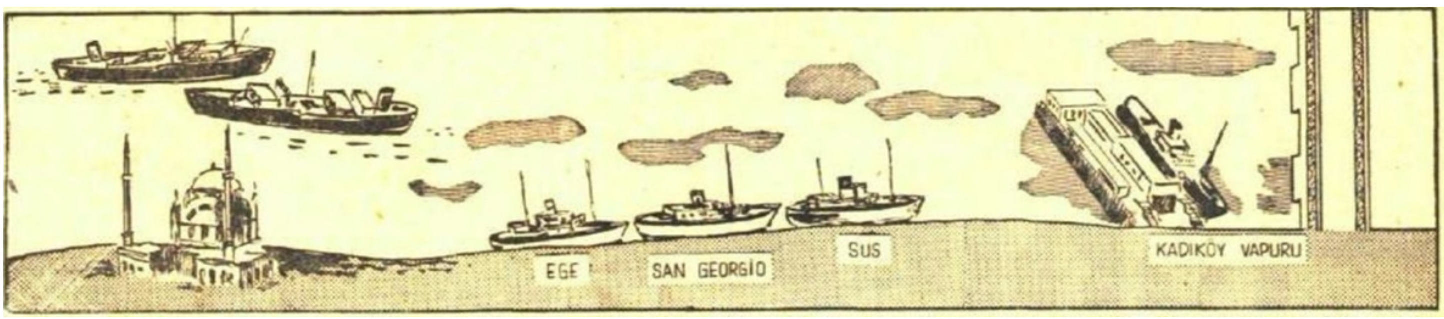


Figure 10. The illustration of the Lutsk-Kransky Oktiyabr accident published on daily newspaper Cumhuriyet on 03/03/2019

Figure 11. The dry cargo vessel Kransky Oktiyabr

Table 3. M/V Kransky Oktiyabr/Mt Lutsk ship characteristics

	M/V Kransky Oktiyabr (North-Bound)	**M/T Lutsk (South-Bound)**
Date-Place Built	23/05/1963-Soviet Union (Kherson Shipyard)	08/05/1964-Ishikawajima Harima Shipyard, Aioi (Japan) No.619
Owner	Black Sea Shipping Company	Black Sea Shipping Company
Type & Flag	General Cargo-Soviet Union	Tanker-Soviet Union
Pilot Onboard	NO	NO
Cargo	Fully Laden Sugar in bags-15.500 tons (From Cuba)	Fully laden- Crude Oil 35.000 tons
Dimensions (Lxbxd)	169.91×21.85×12.9 m	207.04 × 27.06 ×11.08 m
Tonnage	11206 GT	23110 GT
Max.Speed	18.2 Knots	16.5 Knots
Engine&Propeller	Steam Turbine, Single Screw	Steam Turbine, Single Screw

b. **After- accident era:** The Prosecutor's Office opened proceedings against the captains of Russian ships under Article 383 of the Turkish Penal Code. This article (ın that time) contained the following provision: "A person shall be sentenced to 30 months' imprisonment and a fine of up to 100 pounds if he or she causes a fire or explosion or destruction of the public hazard due to precariousness and carelessness or inexperience in art, inexperience in the profession, or in breach of rules or regulations." Both vessels were taken under arrest. Soviet representatives of the Black Sea Shipping Company accepted the guarantee payment. The Kransky Oktiyabr was released first, and after the payment of 29 Million 675 Thousand Turkish Liras as guarantee, the tanker Lutsk was released on 1 May 1966 and the ship left Istanbul for Odessa on that day.

d. **Accident analysis:** The following are the findings with regard to this accident (Applying the same criteria):

1. **Navigational errors:** The Kranskiy Oktiyabr followed the appropriate course in compliance with the Istanbul Port Regulation. The other vessel involved in the collision; the tanker Lutsk did not follow the appropriate course in the same regard. After the first sight, both vessels came to a head-on situation when Lutsk altered her course to port and Kransky Oktiyabr altered her course to starboard. In this situation, both vessels seem to be confused between the rule imposed by Port Regulation and the one imposed by Collision Regulations on head-on situations. In fact, Rule 18 of the 1960 version of International Regulations for Preventing Collision (Revised in 1972) which came in to force in 1965, ordered that "When two vessels approached each other from reciprocal or nearly reciprocal courses so as to involve risk of collision, each shall alter her course to starboard" (Same rule was derived into 1972 COLREG's as Rule 14) (Lameijer et al, 2004). To comply with the Collision Regulations; each vessel should alter her course to starboard; Kransky Oktiyabr did this but Tanker Lutsk did not. On the other hand, to comply with the İstanbul Port Regulation Article 26, both vessels should alter their courses to port, from this perspective, Lutsk complied with the regulations and Kransky Oktiyabr did not. It is apparent that there was a conflict between two regulations as detailed above. This confusion might have played a critical role in the Lutsk-Kransky Oktiyabr accident.
2. **Pilot onboard:** Both vessels, neither Kransky Oktiyabr nor Lutsk, did not use pilot for their passage through the Strait of Istanbul.
3. **Meteorological/morphological difficulties**: There was not any noteworthy conditions with regard to current or visibility. Visibility was good and currents were weak.
4. **Speed in the strait:** Speed of both vessels might have played a role in the accident from the perspective of preventing it, but this was marginal. Kransky Oktiyabr reduced her speed, which negatively affected her maneuverability, but on the other hand, this action reduced the impact of the collision. On the other hand, Lutsk did not reduce her speed, if she did, the collision could have been avoided.
5. **Left-hand side navigation and crossing areas**: After the accident, Captain Tolochko and Captain Kozyr sent telegrams to their main offices, in other

words, to Black Sea Shipping Company. In their telegrams, the captains blamed each other for being responsible of the accident. However, Minister of the Navy, V. G. Bakaev, had a different viewpoint. According to him, none of the captains was responsible of the accident. Responsible was the rule, which required a crossing from Left-hand side navigation to right-hand navigation near exit. This view, however, has a truth in it. In a narrow Strait, crossing of the lanes apparently increases the risks and leads to confusion. Remembering that at 1966 there was no shore-based assistance providing information to ships about the opposite traffic and their intention, this crossing area played a decisive role in the collision between Kransky Oktiyabr and the Lutsk. As it was detailed in the navigational errors section above, left hand navigation and crossing areas might have confused both captains with the conflicting Collision Regulations rule regarding the head-on situations.

M/T Independenta-M/V Evriali Collision

One of the world's largest marine accidents occurred on November 15, 1979 in the Southern approach of the Strait of Istanbul. Romanian flagged tanker, M/T Independenta, which had a capacity of 147.631 DWT, was a new-built vessel with only two and a half years of age. Nicolae Ceausescu wanted, in the 1970s, to become independent from Russia, in terms of oil. Impressive-sized vessels had begun to be built. This is how "Independenta", the largest ship ever built in Romania came out, the "admiral" of the commercial fleet, a mioritic "Titanic", the first "child" from a list of high tonnage vessels, with swimming pool, hall of games and built 700 kilometers of welding, 150 kilometers of cables, 100 kilometers of pipes are just some of the data of the mastodon project. The M/T Independenta was a 283 Meters in length, 46 meters in breadth supertanker with 22-meters of moulded depth, excluding the superstructure. She was launched in May 27, 1977 on a ceremony in which Elena Ceausescu, wife of Romania's President Nicolae Ceaușescu's wife, did not succeed in breaking the champagne bottle against the ship's hull, which accepted a sign of bad luck for the vessel.

Figure 12. Elena Ceausescu trying to break the champagne bottle, a bad sign for the ship's fate

On her last voyage, which was terminated by a tragic accident, Independenta was on the way from Libya to Constanta, with a cargo of 93800 tons of Es-Sider crude oil. The vessel also had 260 tons of heavy fuel oil bunkers on-board. M/T Independenta was not fully loaded (Her tanks were 2/3 loaded, as the full capacity was 147.631 tons; this incomplete load of crude-oil in the tanks probably caused gas accumulation in the tanks which played a role in the accident). M/T Independenta was under command of Captain Dorinel Grigore Mihai, born on 01 May 1944 in Candesti, Buzau in Romania, 35 years old at the time of accident. In fact, Captain Constantin Preda was the usual Captain of the tanker, who was onboard during the all previous voyages, then left on holiday; Captain Dorinel Mihai replaced him for the last voyage. Being a captain onboard, an oil tanker was not Captain Mihai's best choice, and he had already resigned on 2nd of November and his resignation would take effect after 15 days, on the 17th of November. According to his crew, Capt. Mihai was an enthusiastic person, as a hobby, he often played violin and "ballad" by Ciprian Porumbescu, the famous Romanian composer, was his favorite (Mihailescu, 2009). But that did not mean that he was not a prudent seaman. He was diligent to comply with safety rules and recommendations. He required a pilot on his arrival at the entrance of Strait of Istanbul. In fact, Independenta arrived in Istanbul anchorage on 14 November at 16:00, but as nighttime passage was not permitted for large tankers of this size, the tanker went to anchorage to wait until the early morning on the next day. Time to enter was given at 07:00 Local Time on 15 November 1979, a rendez- vous to which Capt. Mihaiarrived two hours earlier, at 05:00 local time. He was recommended to slow down and wait for the pilot near the entrance. At that time, there was no land-based traffic management system established in the Strait of Istanbul, traffic management was partly carried out by the pilot stations and pilots themselves. The pilot station at service for Strait of Istanbul then was located at Harem district, which was relocated to More-Southerly İnciburnu area about 20 years later, due to its location being too much inside of the Strait, which had used to deteriorate service availability to vessels require pilot in relevant pilot boarding area. On 15 November 1979, in Istanbul, early morning, state of the sea was calm, but visibility was restricted, around 1.5 nautical miles. The sunrise time on that day was 06:51, according to (-2) GMT calculation, for this reason, entrance time of tanker Independenta was fixed at 07:00. At the other end of the Strait, another ship, Greek freighter Evriali entered the Strait at 04:00 and picked up the pilot near Kavak Point. The pilot's name was Capt. Dinçer Sümerkan. Under the pilotage of Capt. Sümerkan, freighter Evriali safely passed through the Strait arrived near Kızkulesi, around which Capt. Sümerkan left the ship; time was 05:15, just twenty minutes before the collision. This was the common practice at that time, leaving the vessels right after the Kızkulesi area, so what Capt. Sümerkan did was not something exceptional, but there was one issue which was noteworthy, if not exceptional, that was a large tanker approaching and visibility was not so good. Capt. Sümerkan explained the whole situation on a TV Interview conducted by Romanian Digi 24 TV in 2017 as follows: "It was very dark, there was fog, the visibility was about 1,5 nautical miles. Independenta was 4 nautical miles from us at the last radio talk. In the morning around 4 o'clock, I had boarded the Evriali ship, at 5:15 I disembarked and headed for pilot station at Harem, and at 5:35 I heard that Evriali collided with the Romanian ship. As far as I learned, instead of heading to course of 260 to the starboard, she altered about 30 degrees more to port from existing course, to 160, to the port side. But I don't understand why he did that". At 5.15 Capt. Dinçer Sümerkan, pilot of M/V Evriali, left the vessel after a beam of Kızkulesi and according to him, he left the ship heading to 190 degrees, which is the usual course after passing Kızkulesi until which the course should be 235 degrees. If Evriali had continued on this course, the collision would not have occurred. Evriali came to a head-on situation with the tanker, and altered her course to port, directly towards the tanker. On the reciprocal course, the Tanker altered her course to Starboard. On Evriali, Captain Alekos Adamopoulos was 29 years old. According to daily newspaper Cumhuriyet, on 19 November 1979 issue, it was claimed that both captains had a conversation on VHF minutes before the accident. According to the news, on this conversation, Captain Mihai, warned Captain Adamopoulos to pass "port-to-port" and this request was approved by the Greek freighter. However, in order to comply with this agreement, Captain Adamopoulos should alter the Freighter's course to starboard, which he did the contrary. Altered to port. Reason for this, according to newspaper, could be a misunderstanding between two captains. Captain Adamopoulos could have understood the proposal to pass "Port-to-Port" as, "hard to port!" and give this command to the helmsman. On the Independenta, some seafarers were on deck, with the camera, because the Strait and the city of Istanbul were wonderful. But there was something going wrong, in the twilight, a freighter was very close, a few hundred meters. Independenta insistently altered to starboard, Evriyali insistently altered to port, almost in vertical course, as they got closer. It was 05.33, right before the collision, Captain Dorinel Mihai give five short signals with the whistle, which was heard all seafront houses in Kadıköy and Kabataş (Özözlü, 2018). Two minutes after

Evriyali's stem, close to the starboard bow, crashed into Independenta on the port side, on No. 3- No. 4 tanks area, just in front of the accommodation. Especially No.4 tanks were critical area because these tanks did not contain crude oil, but gasoline. Soon after the crash, crude oil started to pour out from the tanks and sparks due to metal-to-metal friction, pouring crude oil-or gas- caught fire, and afterwards, there was a huge explosion, due to which, all the windows glasses in a 6 km diameter in Istanbul city were broken. Abandon ship bells rang on board Independenta. Chief Engineer of M/T Independenta, Sorin Mihăilescu, described the tragic moments as follows (Cumpana, 2006): "I went on the bridge. The first who saw the Evriali ship was Commander Mihai Dorinel. He was focused and seemed a little anxious. I also saw the ship heading in the opposite direction towards us. At one point, the commander noticed the wrong direction. The foreign ship did not sail "on the starboard side" to show us her port side, as was the rule. We all thought he was a little off the road and that he would soon correct his course. Our commander put his handset on the radio and sent them to come «port to port», that is to pass parallel with us and to see the red light from his port side, as he saw the one from our port. It is an elementary rule of sea navigation at night. No one answered. Later, I learned that the ship, though passing through the Strait, had no pilot on board, the commander was not in command, and permanence was ensured only by the ordinary cart, an officer, a helmsman and a sailor. I never understood what was their intention. Maybe they bet on the routine, maybe they didn't even see us, maybe they were disconnected because they were just getting out of trouble, maybe they were worried about something else. There were some rours that they had women on board or that they were doing "business" with the locals who were fishing in the area. Mihai Dorinel kept calling him over the radio. The other ship was silent. It was now 5.20. The foreign vessel continued to proceed towards us. We were beginning to distinguish their profile and dimensions. It was a cargo of about 5,000 DWT. Later I found out that it was called "Evriali", it was private property, under the Greek flag and carrying steel product. Within minutes, our commander's calls began to become more and more agitated. I was worried about his voice. At that moment, the whistle of our ship came into operation; their thick and frightening scream was a desperate attempt to warn the stranger of our presence and imminent danger. But the ship in front of us gave no sign, instead, it changed course and turned perpendicular to ours. Due to the size and weight of the "Independence" tanker, any avoidance manoeuvres were impossible. The "Evriali" had come close enough to see its light from the starboard, and not from the port as normal. There was nothing left to do. It was now 5.30. From a defense instinct, I pulled out the flashlight and tried to signal. The killer ship continued on its way and came directly toward us, almost perpendicular. It was now 5.35. «Independenta» received the blow in the port, near tanks 3 and 4. There was a deafening thud and a loud metallic noise. A roar of torn and crushed iron mixed with the desperate screams of the people. The shock of the collision was not felt. Independenta was 150,000 tons, it was huge, and the Greek one about 7,000. There was no explosion at the time of the impact, but due to the metal-to-metal friction, there were strong sparks, which ignited the oil stream that had started to pour through the crack produced by the blow. Then the first explosion took place. A frightening thunder swept toward the vault of the sky and then crashed upon us, bursting with its shock wave. A huge column of apocalyptic red, yellow and orange flames tore through the night's shattering darkness. Magnificent black and smoky hummingbirds, lit by flames, camped over us and the ship. There are no real grounds for rumours and speculations regarding the inert gas system that it was defective, that it did not work. I affirm with all responsibility that this system worked. The fire broke out because of the sparks that occurred at the time of the collision. Then remember that tank no. 4, although it did not contain oil, it had gas."

The explosions after the accident were so huge that the glasses on the windows of the Kadıköy district and the glasses of the Topkapı Palace were broken, although the entrance took place quite a distance. In Istanbul only, 10 million Turkish Lira (about $ 250 thousand) had to be spent for the repair of the windows. Oil spilt from the tanker spread over the sea in an area of 1.5 miles and burnt, occasional explosions occurred in the tanker, the people of Istanbul experienced a hell of fear. A dark smoke spread all over the city. M/T Independenta, which was 3 km away from the Topkapı Palace at the collision, drifted closer to the city out of command towards the shores of Kadıköy, on the other hand, the oil continued to be burned and the ship was bursting. Drifted up to four hundred meters before the Haydarpaşa breakwater, the tanker was stranded there and then split in two accompanied with a big explosion. The flames from the exploding tanks were rising up to 50 meters in the sky, and the thick, toxic and smoke fumes collapsed like blankets over the city.

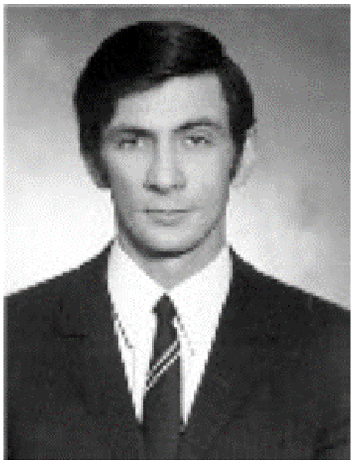


Figure 13. Captain Dorinel Grigore Mihai, the unfortunate captain of the Independenta tanker, was among the 11 unfortunate sailors whose body was not found after the accident (Cumpana, 2006).

Figure 14. Capt. Alex Adamopoulos, Captain of the Greek freighter Evriali (Photo from Turkish Daily Newspaper Milliyet, Published on 16.11.1979).

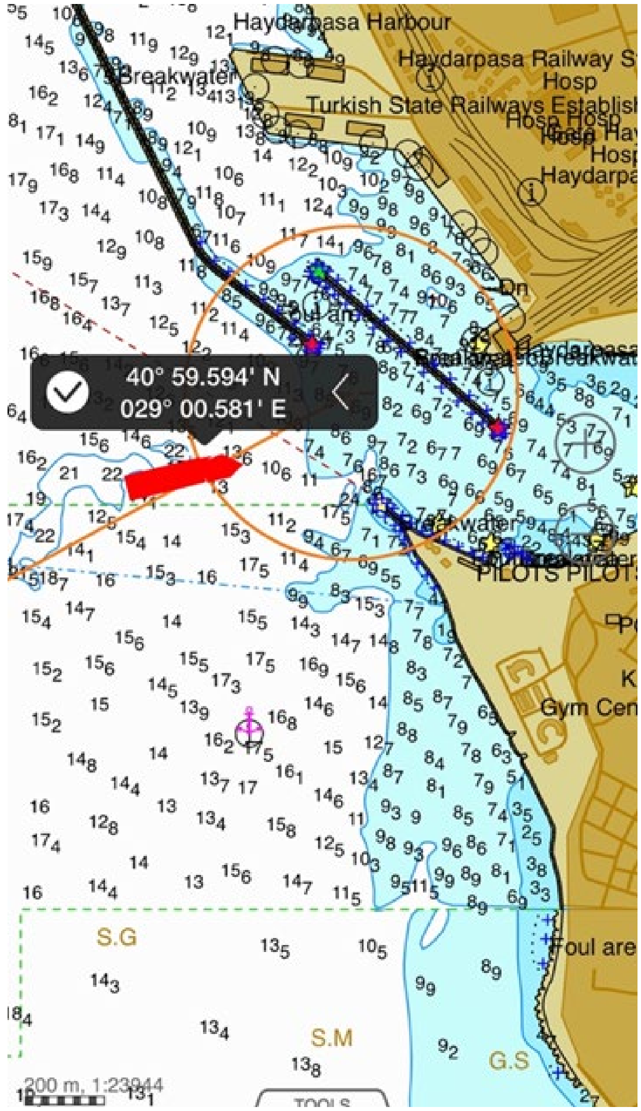


Figure 15. The location where Independenta went aground

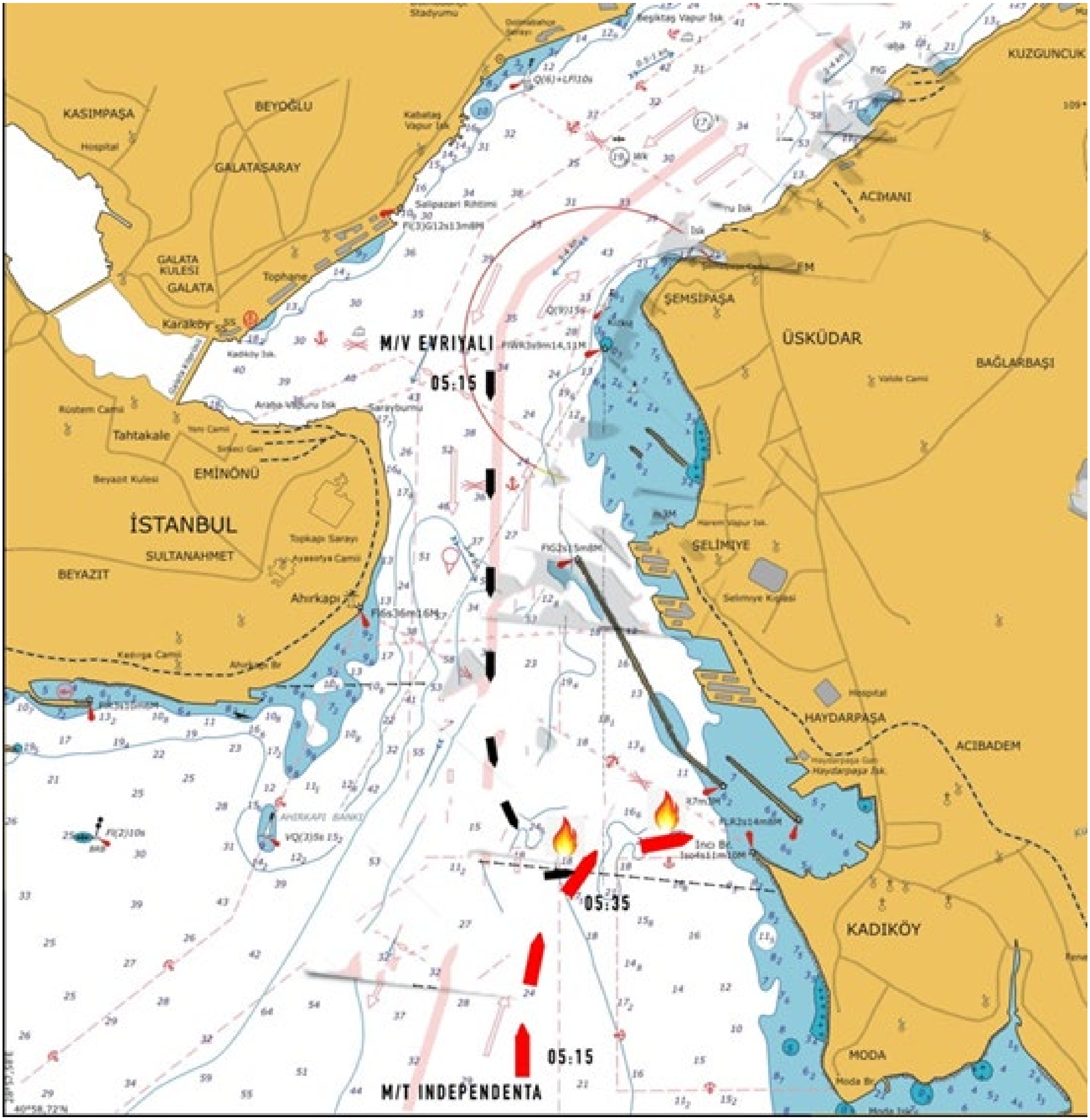


Figure 16. Estimated collision tracks and site of collision of M/V Evriyali and M/T Independenta

Figure 17. Independenta aground and on fire right before the Haydarpaşa breakwater. Source: https://onedio.com/haber/11-maddede-bogaz-daki-en-buyuk-tanker-faciasi-independenta-515265

Table 4. Mt Independenta/Mv Evriali ship characteristics

	M/T Independenta (North-Bound)	**M/V Evriali (South-Bound)**
Date-Place Built	23/05/1963- Santierul Naval Constanta, Constanta	1971- Astilleros Armon Gijon - Gijon, Spain
Owner	Navrom Shipping Co., Constanta	-
Type & Flag	Crude Oil Tanker-Romania	General Cargo-Greece
Pilot Onboard	NO (Waiting for)	NO (Just dropped)
Cargo	Crude Oil-2/3 Laden-93800 tons (from Es Sider/Libya)	Fully laden- Steel Product 7500 tons (from Zhdanov/Ukraine)
DIMENSIONS (Lxbxd)	283 × 46 × 22.6 m	118 × 17 × 7.5 m
Tonnage	88690 GT	5298 GT
Max. Speed	16 Knots	14 Knots
Engine & Propeller	1 Diesel Engine, Single Shaft, Single Screw	Diesel Engine, Single Screw

There were 45 crewmembers in Independenta and 33 in Evrialy. All of the crew of the Greek freighter survived with a great chance, while 42 of the 45- total crewmembers of the unfortunate Romanian tanker lost their lives; only 3 of them survived. Among 42 crew of Independenta who lost their lives, it was not even possible to find the bodies of 11 of them, including the Captain, Dorinel Mihai.

After the accident, the freighter Evriali was towed towards the Ahırkapı anchorage area and the fire onboard the freighter was taken under control at this location. But the same could not be said for the ill-fated tanker M/T Independenta. The total amount of 94600 tons of crude oil that was onboard the giant tanker continued to burn, and tanker could not be approached in close proximity by the firefighting tugs which already had limited capacity of extinguishing range at that period of time, and, as the tanks were partly loaded, this condition made the tanker more prone to explosion due to the accumulation of gas. It was impossible to estimate the ratio of oil that was burned compared to that was spilt into the sea. The oil that was spilt into the sea began to drift towards the southern parts of the Sea of Marmara under the influence of the currents flow and caused extensive ecological damage. Thousands of dead fish went ashore on the coasts of the Anatolian part.

Surface bombs were used near the tanker’s wreck, in order to keep the remaining oil on-board, the tanker, while the extinguishing boats sought to control the flames by squeezing detergents and chemicals. The fire onboard the tanker Independenta continued for several weeks, and the ship's wreck moved deeper into the bottom of the sea with ongoing explosions. The remaining ashes mixed with oil generated thick bunches which accumulated over Moda district at the Asian part of the city and led to massive air pollution. On December 6, 21 days after the accident, the fire was still going on and there was a huge explosion this day. In this explosion, the flames rose up to 350 meters towards the sky. The top of the sea became a hell of a fire and the flame began to fall out of the sky over the city. After this big explosion, the flames began to decrease relatively and on December 14, almost 1 month after the accident, the fire went out on its own and completely extinguished.

a. **After- accident era:** The Captain of Greek flagged ship Evriali, Capt. Alekos Adamopoulos and 7 crewmembers were arrested upon a court order after the accident. The accused were attributed to the crimes of 'carelessness, negligence and non-compliance with international navigation rules'. It was also among the accusations that the safety of Istanbul had been jeopardized and the death of 43 sailors in the Romanian tanker. The trial proceedings lasted 7 months and Captain Alekos Adamopoulos was given a 20-month sentence. However, taking into account the length of his detention at the site of Sağmalcılar Prison and his good standing in court, this penalty was converted into a fine of 850 dollars and the captain was released. The wreckage of the tanker remained before Haydarpaşa port district for many years and continued to pose a risk for local traffic. The local ferry Hürriyet allided with the wreck on 19 April 1983 and had to be repaired at the shipyard. The task of wreck removal was given to the Navy, and subsequently Navy overhanded this job to a private sub-contractor company. In mid-November 1983, 8000 tons of wreck was removed, while the contractor firm went bankrupt. In 1986, a tender was won by Mr. Celal Sadıkoğlu for the removal of the wreck. The remaining parts of the wreck were taken to the recycling facilities in Aliağa district of Turkey on the Aegean Coast and finally, 7 years later, Istanbul got rid of this nightmare. The Romanian tanker M/T Independenta's hull and engine insurance cover was $ 40.26 million. Evrialy, whose value was only 2.5 million USD, was also heavily damaged by collision and fire. There was severe damage to the starboard and the stem of Evrialy. Evrialy was sold after 6 years at Tuzla shipyards on 23 June 1986, and her fate ended up at Aliağa ship recycling facilities after this sale.

b. **Accident Analysis:** The following are the findings with regard to this accident (Applying the same criteria):

   1. **Navigational errors:** According to the information in hand, The Greek freighter, Evriyali is responsible of the accident. Evriali followed the wrong course and did not carry out the proper manoeuvring in compliance with COLREG’s. After the pilot had left the freighter, there should have been a head-on situation, due to which, Independenta altered her course to starboard and Evriali altered her course to port. Altering the course to Port in a head-on confrontation was used to be valid for Istanbul Port Regulation in the certain areas of Strait of Istanbul, as explained above. But not in this part of the Strait. However, it is crucial to know that whether the navigational team members onboard Evriali were aware of this fact. As the Collision Regulations ordered to

alter the course to Starboard and Istanbul Port Regulations ordered to alter the course to Port in head-on situations, and remembering that Evriali followed the Port side of the Strait during the large portion of her passage in the Strait and crossing to starboard section from the port in front of Kızkulesi had just completed before the pilot was left, situational awareness of Evriali navigational team was -most probably- lost right before the accident.

2. **Pilot onboard:** Both vessels, neither Independenta nor Evriali, did not have pilot onboard at the time of the accident. Evriali had just dropped pilot 20 minutes from the accident and Independenta was waiting the pilot to arrive.
3. **Meteorological/morphological difficulties**: The conditions were moderate, with visibility restricted to 1.5 miles. Moderate NE winds were prevailing during the time of accident.
4. **Speed:** Speed of both vessels before of at the time of accident were unknown. But, from the data known, it can be said that speed of Evriali was more compared to Independenta. According to estimation, speed of Evriali was around 8 Knots and speed of Independenta was about 4-5 knots right before and during the time of the accident.
5. **Left-hand side navigation and crossing areas**: As explained above, confusion due to Left-hand side navigation and crossing areas might have played a role and might be attributed to this accident as the behaviour of Evriali-ship responsible of the accident- cannot be explained in another way, if not a gross negligence or intent was the case. Both vessels came into a head-on situation in which Evriali insistently altered her course to port and Independenta insistently altered her course to starboard. The area was outside of the boundaries of left-hand side navigation application area in the Strait of Istanbul. But to what extent the Captain of Evriali was aware of this situation? Having complied with the left-hand side rule at most part of the Strait, even through the advices of a pilot, might have created a confusion.

Conclusions

The Independenta-Evriali accident was a game changer in the Strait of Istanbul. After this accident, discussions were ignited regarding the safety of the Straits. At the post-accident era, the 1936 Montreux Convention was there to blame, which brought a ban on the compulsory pilotage. And, Left-hand side navigation system was discussed. However, this issue was controversial. Some technical experts, including the pilots, said that Left-hand side navigation was compatible with the Strait of Istanbul and right-hand navigation was incompatible and would increase accidents, let aside eliminating them (Turkish Daily Newspaper Milliyet, 02 May 1982). Some veteran pilots said that although Left-hand side navigation had some advantages by being more compatible with the structure of the Strait, crossing lines at two ends of Strait could generate accidents (Barlas, 1979). In the researches carried out by the technical committee established within the Ministry of Transportation, a conclusion was reached on making changes in the maritime traffic order by providing navigation, life, property and environmental safety in the Straits. In this conclusion, the idea of changing the 'Left-hand side Navigation Order' to the 'Right-Hand Navigation Order' and controlling the traffic in the Strait of Istanbul was adopted. In May 1981, this report was submitted to the National Security Council (MGK). Report included the following statements:

“While entering the Strait of Istanbul from the Marmara, a vessel navigating from the right side of the Talveg line according to international rules has to pass from right to left in the area of Kızkulesi-Ortaköy Mosque line where traffic is the most intense, crossing again from Umurbank Lighthouse and leaving the Strait. Ships that will pass from the Black Sea to Marmara also apply the transitional order in the opposite direction. Within the boundaries of the Port of Istanbul, the current crossing order, which is applied in contradiction with international rules and increases the possibility of accident due to crossing areas, should be abolished and the right navigation order in accordance with international rules should be applied.” (Turkish Daily “Milliyet” newspaper, 11.10.1981).

This report, prepared by the Ministry of Transportation and submitted to National Security Council, clearly set forward the conflict of National rules with the International rules. In all of the three major accidents, there were head on situations of vessels in reciprocal courses. In addition, in three of them, one of the vessels involved in the accident altered her course to starboard while the other altered her course to port. As mentioned before, action of one vessel complied with the local rule and action of the other complied with the international rule.

After the report of Ministry of Transport, the necessary amendments were made to the Istanbul Port Regulation prepared by the Ministry of Transport and sent to the Council of State for examination. After being reviewed by the Council of State, it was published in the Official Gazette after being approved by the decision of the Council of Ministers with the number of 8/4538 dated 9 April 1982. Article 10 of the Istanbul Port Regulation, which entered into force on 1 May 1982, included:

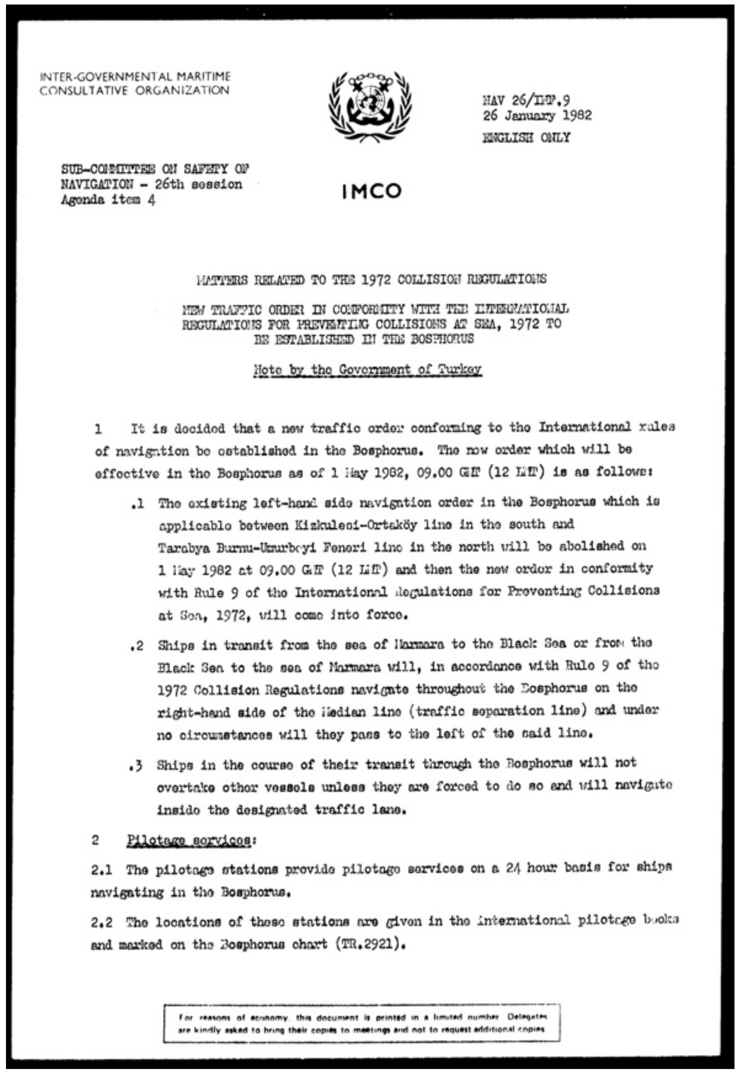
"The sea area between the line connecting the Türkeli Lighthouse and Anadolu Lighthouse in the North and the Ahırkapı Lighthouse in the South and Kadıköy İnciburnu Lighthouse in the South, consisting of two traffic lanes and a center line separating these lanes for north and south directional traffic suitable for the passage, is called the passageway for the Strait."

According to this new system, ships crossing from the Black Sea towards the Sea of Marmara and vice versa, would pass through, from the entrance of the Strait to the exit, using the right lane in accordance with the International Rules for the Prevention of Collision at Sea.

Turkish Government, also sent an information paper to the Intergovernmental Maritime Consultative Organization (IMCO), NAV/26-Inf.9 on 26 January 1982 including a notification that the Left-hand side side navigation partly used in the Strait of Istanbul was to be abolished and new traffic order would be in full compliance with COLREG Rule 9, which regulates the traffic in narrow channels. Turkey's paper, NAV/26-Inf.9, included the following statements:

"It is decided that a new traffic order conforming to the International rules of navigation be established in the Bosphorus. The new order, which will be effective in the Bosphorus as of 1 May 1982, 09.00 GMT (12 LMT) is as follows:

1. The existing Left-hand side navigation order in the Bosphorus which is applicable between Kizkulesi-Ortaköy line in the south and Tarabya Burnu-Umurbeyi Feneri line in the north will be abolished on 1 May 1982 at 09.00 GMT (12 LMT) and then the new order in conformity with Rule 9 of the International Regulations for Preventing Collisions at Sea, 1972, will come Into force.
1. Ships in transit from the sea of Marmara to the Black Sea or from the Black Sea, to the sea of Marmara will, in accordance with Rule 9 of the 1972 Collision Regulations navigate throughout the Bosphorus on the right-hand side of the median line (traffic separation line) and under no circumstances will they pass to the left of the mid line.
2. Ships in the course of their transit through the Bosphorus will not overtake other vessels unless they are forced to do so and will navigate inside the designated traffic lane."



INTER-GOVERNMENTAL MARITIME CONSULTATIVE ORGANIZATION

IMCO

NAV 26/INF.9
26 January 1982
ENGLISH ONLY

SUB-COMMITTEE ON SAFETY OF NAVIGATION - 26th session
Agenda item 4

MATTERS RELATED TO THE 1972 COLLISION REGULATIONS

NEW TRAFFIC ORDER IN CONFORMITY WITH THE INTERNATIONAL REGULATIONS FOR PREVENTING COLLISIONS AT SEA, 1972 TO BE ESTABLISHED IN THE BOSPHORUS

Note by the Government of Turkey

1 It is decided that a new traffic order conforming to the International rules of navigation be established in the Bosphorus. The new order which will be effective in the Bosphorus as of 1 May 1982, 09.00 GMT (12 LMT) is as follows:

.1 The existing left-hand side navigation order in the Bosphorus which is applicable between Kizkulesi-Ortaköy line in the south and Tarabya Burnu-Umurbeyi Feneri line in the north will be abolished on 1 May 1982 at 09.00 GMT (12 LMT) and then the new order in conformity with Rule 9 of the International Regulations for Preventing Collisions at Sea, 1972, will come into force.

.2 Ships in transit from the sea of Marmara to the Black Sea or from the Black Sea to the sea of Marmara will, in accordance with Rule 9 of the 1972 Collision Regulations navigate throughout the Bosphorus on the right-hand side of the Median line (traffic separation line) and under no circumstances will they pass to the left of the said line.

.3 Ships in the course of their transit through the Bosphorus will not overtake other vessels unless they are forced to do so and will navigate inside the designated traffic lane.

2 Pilotage services:

2.1 The pilotage stations provide pilotage services on a 24 hour basis for ships navigating in the Bosphorus.

2.2 The locations of these stations are given in the international pilotage books and marked on the Bosphorus chart (TR.2921).

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Figure 20. Informational note of Turkish Government to International Maritime Organization regarding the new traffic order in the Strait of Istanbul which took effect on 1st of May, 1982

The new system started to be applied by 1 May 1982. In these days, 70 ships were passing per day, almost half of the number of ships that are passing today. New system increased the number of ships use pilot, which increased around 50 percent. In the new system, right hand navigation, as it was called, the first vessel to pass was the Greek flagged Dalia A., a vessel departed from Constanta, Romania and bound for Alexandria, Egypt. The first ship to pass in the new order was boarded by four Turkish Ministers including the Transportation Minister Mustafa Aysan and a plaque was given to Ship's master, Capt. Vutinas. That was the end of an era in the Strait of Istanbul, which was, to date, only sea area in the world, in which Left-hand side navigation was being used.

This was also the end of the era in which conflicting national and international rules in the Strait of Istanbul were in practice. With the establishment of Colreg Rule 9 in the Strait of Istanbul, which regulates the navigation in narrow channels, and abolishment of the left-hand side navigation, the navigational system in the Strait of Istanbul became compliant with the International system.

Compliance with Ethical Standard

Conflict of interests: The authors declare that for this article they have no actual, potential or perceived conflict of interests.

Ethics committee approval: There is no need ethics committee approval.

Financial disclosure: The authors declare that for this article there are no financial support.

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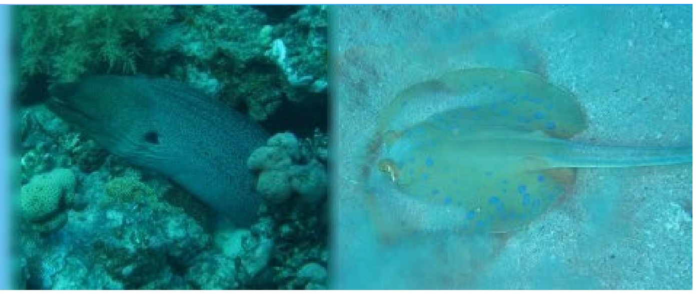

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Key words (indexing terms), normally 3-6 items

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References

Tables

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Original Articles: This is the most important type of article since it provides new information based on original research. The main text should contain Introduction, "Materials and Methods", "Result and Discussion" and Conclusion sections.

Statistical analysis to support conclusions is usually necessary. Statistical analyses must be conducted in accordance with international statistical reporting standards. Information on statistical analyses should be provided with a separate subheading under the Materials and Methods section and the statistical software that was used during the process must be specified.

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Table 1. Limitations for each manuscript type

Type of manuscript	Page	Abstract word limit	Reference limit
Original Article	≤25	180	40
Review Article	no limits	180	60
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Tables should be included in the main document, presented after the reference list, and they should be numbered consecutively in the order they are referred to within the main text. A descriptive title must be placed above the tables. Abbreviations used in the tables should be defined below the tables by footnotes (even if they are defined within the main text). Tables should be created using the "insert table" command of the word processing software and they should be arranged clearly to provide easy reading. Data presented in the tables should not be a repetition of the data presented within the main text but should be supporting the main text.

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All references, tables, and figures should be referred to within the main text, and they should be numbered consecutively in the order they are referred to within the main text.

Limitations, drawbacks, and the shortcomings of original articles should be mentioned in the Discussion section before the conclusion paragraph.

References

Reference System is APA 6th Edition

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The APA style calls for three kinds of information to be included in in-text citations. The **author's last name** and the work's **date of publication** must always appear, and these items must match exactly the corresponding entry in the references list. The third kind of information, the page number, appears only in a citation to a direct quotation.

....(Crockatt, 1995).

Direct quote from the text

"The potentially contradictory nature of Moscow's priorities surfaced first in its policies towards East Germany and Yugoslavia," (Crockatt, 1995, p. 1).

Major Citations for a Reference List in Table 2.

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Accepted manuscripts are copy-edited for grammar, punctuation, and format. Once the publication process of a manuscript is completed, it is published online on the journal's webpage as an ahead-of-print publication before it is included in its scheduled issue. A PDF proof of the accepted manuscript is sent to the corresponding author and their publication approval is requested within 2 days of their receipt of the proof.

Table 2.

Material Type	Reference List/Bibliography
A book in print	Baxter, C. (1997). *Race equality in health care and education.* Philadelphia: Ballière Tindall, p. 110-115, ISBN 4546465465
A book chapter, print version	Haybron, D.M. (2008). Philosophy and the science of subjective well-being. In M. Eid & R. J. Larsen (Eds.), *The science of subjective well-being* (p. 17-43). New York, NY: Guilford Press. ISBN 4546469999
An eBook	Millbower, L. (2003). *Show biz training: Fun and effective business training techniques from the worlds of stage, screen, and song*. p. 92-90. Retrieved from http://www.amacombooks.org/ (accessed 10.10.2015)
An article in a print journal	Carter, S. & Dunbar-Odom, D. (2009). The converging literacies center: An integrated model for writing programs. *Kairos: A Journal of Rhetoric, Technology, and Pedagogy,* 14(1), 38-48.
Preview article in a journal with DOI	Gaudio, J.L. & Snowdon, C.T. (2008). Spatial cues more salient than color cues in cotton-top tamarins (*Saguinus oedipus*) reversal learning. *Journal of Comparative Psychology,* https://doi.org/10.1037/0735-7036.122.4.441
Websites - professional or personal sites	*The World Famous Hot Dog Site.* (1999, July 7). Retrieved January 5, 2008, from http://www.xroads.com/~tcs/hotdog/hotdog.html (accessed 10.10.2015).
Websites - online government publications	U.S. Department of Justice. (2006, September 10). Trends in violent victimization by age, 1973-2005. Retrieved from http://www.ojp.usdoj.gov/bjs/glance/vage.htm (accessed 10.10.15).
Photograph (from book, magazine or webpage)	Close, C. (2002). *Ronald.* [photograph]. Museum of Modern Art, New York, NY. Retrieved from http://www.moma.org/collection/object.php?object_id=108890 (accessed 10.10.2015).
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