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# Erythrocytic Nuclear Abnormalities and Leucocyte Profiles of Asian Seabass (*Lates calcarifer*) Exposed to Polluted Seawater

(Keabnormalan Nukleus Eritrosit dan Profil Leukosit Siakap Asia (*Lates calcarifer*) Terdedah kepada Air Laut Tercemar)

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ABSTRACT

Close to industrial activity and a major seaport in the Gulf of Thailand, Sichang Island has frequently suffered from pollution and oil spills. However, the environmental health status of the surrounding waters is relatively unknown. Between December 2017 and January 2018, we reared ninety Asian seabass (*Lates calcarifer*) in cage nets off the island and sampled blood to investigate nuclear abnormalities in erythrocytes and build leucocyte profiles. The fish were purchased locally and reared in locations where oil spills have been recorded. Environmental parameters were within the range of standard values. Erythrocyte nuclei had segmented, reniform and notched abnormalities; however, they dramatically varied after exposure. At the end of the three-month field experiment, very few micronuclei had been observed. Observed leucocytes were neutrophils, lymphocytes, and monocytes. The percentage of lymphocytes increased considerably, reaching a post-exposure peak at three months. Our data suggest that erythrocytic nuclear abnormalities of Asian seabass could be used as an early warning of toxic pollutants in the marine environment, and as a baseline environmental health indicator for Sichang Island and the surrounding area.

Keywords: Erythrocytes; fish health status; hematology; leucocyte profiling; Sichang Island

ABSTRAK

Terletak berhampiran dengan aktiviti perindustrian dan pelabuhan utama di Teluk Thailand, Pulau Sichang sering mengalami pencemaran dan tumpahan minyak. Walau bagaimanapun, status kesihatan persekitaran perairan sekitar tidak diketahui. Antara Disember 2017 dan Januari 2018, kami menternak sembilan puluh ikan siakap Asia (*Lates calcarifer*) dalam jaring sangkar di luar pulau dan sampel darah diambil untuk mengkaji keabnormalan nukleus dalam eritrosit dan membina profil leukosit. Ikan dibeli secara tempatan dan diternak di lokasi tumpahan minyak direkodkan. Parameter persekitaran berada dalam julat nilai piawai. Nukleus eritrosit mempunyai keabnormalan bersegmen, reniform dan berlekuk; walau bagaimanapun, mereka berubah secara mendadak selepas pendedahan. Pada akhir tempoh percubaan lapangan selama tiga bulan, sangat sedikit mikronukleus telah diperhatikan. Leukosit yang diperhatikan ialah neutrofil, limfosit dan monosit. Peratusan limfosit meningkat dengan ketara, mencapai kemuncak selepas pendedahan selama tiga bulan. Data kami mencadangkan bahawa keabnormalan nukleus eritrosit bagi ikan siakap Asia boleh digunakan sebagai amaran awal bahan pencemar toksik dalam persekitaran marin dan sebagai penunjuk kesihatan alam sekitar asas untuk Pulau Sichang dan kawasan sekitarnya.

Kata kunci: Eritrosit; hematologi; pemprofilan leukosit; Pulau Sichang; status kesihatan ikan

## Introduction

Sichang Island is located near Bangkok, the capital city of Thailand. The island has been promoted as a tourist attraction with easy access to public transport (Wattayakorn & Rungsupa 2012) but industrialization and urbanization have created environmental problems in the area. The increasing amount of municipal wastewater discharged off the island (Wattayakorn & Rungsupa 2012) and oil spills have created a hot spot of environmental problems (Senarat et al. 2021, 2018; Wattayakorn & Rungsupa 2012). One of the major environmental concerns from oil spills is the toxicity of polyaromatic hydrocarbons (PAHs), which are the most toxic component in crude oil and have various negative impacts on the marine environment (Halek & Kavous 2008; Seker 2012). Pollution of the marine environment can pose a threat to various organisms, and although the biomagnification of PAHs has been exhibited in aquatic organisms and at higher trophic levels, including among humans, the effects of PAH pollution on marine ecosystems have not attracted enough attention from government and other public bodies, especially in developing countries (Halek & Kavous 2008; Seker 2012).

Fish health can be assessed in various ways, but analysis of blood cell profiles is considered an effective tool since alterations in blood cell profiles can indicate threats to the immune status, genetic integrity, and overall health of an organism (Cheikyula, Koyama & Uno 2009; Kursa & Bezrukov 2008). The appearance of micronuclei and other erythrocytic nuclear abnormalities in peripheral blood cells of marine fish contaminated with crude oil have been well documented (Barsiene et al. 2006). Cheikyula, Koyama and Uno (2009) associated high numbers of micronuclei and other nuclear abnormalities of the seabream *Pagrus major* with increased concentrations of PAHs. Erythrocytic nuclear abnormalities are also a gauge of water quality and aquatic organism health (Carins, Dickson & Westlake 1975; Rodriguez-Cea, Ayllon & Garcia-Vazquez 2003; Senarat et al. 2021). However, the hematologic status of fish in the seas around Sichang Island has not yet been described. There is a need to assess the environmental health status of the area, especially where human settlements and industrial activities co-exist with fish farms. In this area of Thailand, only the peripheral blood cells and erythrocytic nuclear abnormalities of the java rabbitfish, *Siganus javus*, have been investigated and reported with regard to environmental risk (Sopon et al. 2021). In the present work, we sampled and studied the blood of Asian seabass (*Lates calcarifer*) with a focus on erythrocytic nuclear abnormalities and leucocyte profiles as biological markers of the health status of the local marine environment. Our research data from field studies could provide insights into the environmental health status of this ecosystem. A future comprehensive assessment of the health of *L. calcarifer* in this location could serve as an early sentinel system for the overall ecological health of the region.

## Materials and Methods

The fish used in this study were 30 days old. They were purchased from a commercial farm in the Chonburi province of Thailand and reared solely at Sichang Marine Science Research and Training Station (SMaRT) Chulalongkorn university, which is 20.8 km from a coal mine. At SMaRT, the fish (n=90) were given commercial dry feed pellets twice a day and acclimated in the experimental area for 14 days in sea cages with 14.2 meters deep. At the end of the acclimation period, the field experiment commenced for 14 days, which fish were kept individually in floating baskets. The physico-chemical parameters of the rearing water, including PAHs, were monitored, and recorded throughout the experiment. At 30, 60 and 90 days, thirty fish were collected, and euthanized by rapid cooling (Wilson et al. 2009). Blood from all fish during the observed period was collected from the caudal vertebral vein using a 21G × 1” needle and a 2 mL syringe (NIPRO, Japan), following the standard method of Watson, Baer and Benson (1989). Using the manual wedge technique, blood smears were prepared on glass slides, fixed in methanol for 1 min, air-dried, and stained with Dip Quick for 1 min. The stained slides were washed three times with phosphate-buffered saline at pH 7.4 and mounted in mounting medium. Blood cells were characterized by visual examination of the peripheral blood smear according to the guideline of standard hematology and photographed using a Leica DM 750 light microscope equipped with a 100x oil-immersion lens (Leica, Wetzlar, Germany). Numbers of abnormal erythrocytes (100 cells/fish per slide) and leucocytes (10 cells/fish per slide) were determined, and then calculated as a percentage. Data obtained for the abnormal erythrocytes and leucocytes were tested for normality using Kolmogorov–Smirnov test, using $p < 0.05$ as the threshold for significance. Two-way ANOVA was performed to determine statistical differences between day post-exposures at $p < 0.05$ (GraphPad Prism 5).

## Results and Discussion

### Environmental Factors and Pollutant Monitoring Observation

The physico-chemical environmental parameters including water temperature, pH, and DO were in the range of standard values for marine environmental resources (Pollution Control Department 2017). Sichang Island has a history of oil spills and the level and distribution of PAHs in the sea around the island are known to be derived from crude oil (Nuaklong 2018; Wattanayakorn & Boonperm 2004; Wattanayakorn & Rungsupa 2012). The levels and distribution of PAHs from the surface sediments in Sichang Island, varying from 65.2 to 18,970 ng $g^{-1}$ dw, with a median concentration of 282±3,660 ng $g^{-1}$ dw were observed (Wattayakorn & Boonperm 2004). Nuaklong (2018) continuously showed from the same area that the total PAHs of marine sediments ranged from 29.2 to 961 ng $g^{-1}$ dw, with and the average of 99.9 ± 157 ng $g^{-1}$ dw. Although the marine environment from Sichang Island has been mostly identified, PAH pollutants in fish tissue were not quantified throughout the investigation.

### Hematological Parameter

The close relationship between a fish and its environment is reflected in physiological changes (Braham et al. 2017; Giannetto et al. 2014; Parrino et al. 2018; Sadiqul et al. 2016; Shahjahan et al. 2019) and it is widely acknowledged that abnormalities in blood cells, both in quality and quantity, are associated with environmental stress and pollution (Megarani et al. 2020; Parrino et al. 2018; Shahjahan et al. 2019). Hematological disorders can therefore be used to assess the health of fish and facilitate rapid diagnostics (da Silva Corrêa et al. 2017; Fazio 2019; Fazio et al. 2012; Gabriel, Ezeri & Opabunmi 2004; Parrino et al. 2018).

In the present study, blood samples were collected at 30, 60 and 90-day post-exposure. Hematological profiles were constructed, and proportions of cell types determined. Most of the blood cells were unidentifiable cells with an oval shape, a large eccentric nucleus and distinct acidophilic granules (Figure 1(A)-1(B)). It is probable that these blood cells were azurophil leucocytes, which have usually been associated with squamates and crocodilians (Cooper-Bailey et al. 2011; Gutierrez-Cervantes 2022; Oliveira-Júniora, Tavares-Dias & Marcon 2009; Salakij, Salakij & Chanhome 2002). Similar results have been reported in the rattlesnake *Crotalus adamanteus* (Alleman et al. 1999) and other reptiles (Stacy, Alleman & Sayler 2011). However, some sources have classified azurophils as granulocytes or monocytes that contain azurophilic granules (Campbell 1986; Dotson, Ramsay & Bounous 1995; Montali 1988), a classification supported by Tully and Mitchell (2012). In general, azurophils are considered to be primary granules. In reptiles, they are associated with inflammation, infections (especially bacterial (Jacobson et al. 1997)), and defensive mechanisms (Cooper-Bailey et al. 2011; Oliveira-Júniora, Tavares-Dias & Marcon 2009). In the present study, the number of azurophils significantly increased ($p < 0.001$) at two months post-exposure and significant decreased ($p < 0.001$) at three months (Table 1). This could be a result of increased inflammation (Martínez-Silvestre, Lavín & Cuenca 2011) and long-term physiological irritation or chronic disease (Stacy, Alleman & Sayler 2011).

Thrombocytes were ellipsoid to oval with a purple-stained nucleus and light purple cytoplasm (Figure 1(C)). Similarly, to azurophils, the number of thrombocytes spiked after two months and then dropped off at three months post-exposure. And just as azurophils can be linked to inflammation and immunological defense, so have rising numbers of thrombocytes (da Silva Corrêa et al. 2017). These symptoms might be the result of long-term exposure to toxic pollutants and have previously been proposed as important biomarkers in vertebrates because reductions in their numbers compromise the immune system, increasing the exposure of the organism to stress (Dieterlen-Lievre 1988; Sypek & Borysenko

1988).

Regarding the peripheral blood cell types of *L. calcarifer*, normal erythrocytes were the most abundant. These cells were oval with a compact and central oval nucleus (Figure 1(A)), similar to other healthy fish species (Kousar & Javed 2015; Okomoda et al. 2018; Singkhanan et al. 2019). We found four variations of erythrocytic nuclear abnormality: segmented, reniform, and notched nuclei, and micronuclei during post-exposure (Figure 1(B)-1(G)). However, the number of erythrocytic nuclear abnormalities did not significantly differ during the post-exposure period. Because they differed, it is proposed that these features are considered to be indicators of cytogenetic damage (Pacheco & Santos 2002) and useful biomarkers for ecotoxicological evaluations of environmental pollution (Carrasco, Tilbury & Myers 1990). The appearance of abnormalities in erythrocyte morphology has been proposed as evidence of physiological stress from heavy metal toxicity (Güner & Muranli 2011; Kousar & Javed 2015), genotoxic damage (Kousar & Javed 2015; Summak et al. 2010), high concentrations of PAHs, PCB, and nutrient enrichment (Azevedo et al. 2009; Bicego et al. 2006; Hortellani et al. 2005), and ionizing radiation (Anbumani & Mohankumar 2011). Increases in micronuclei have been directly related to levels of PAH contamination and mercury compounds (Porto, Araujo & Feldberg 2005), mutagenic agents and industrial sewage (Torres de Lemos et al. 2007).

The number of micronuclei could be an excellent tool to assess the genotoxic pollutants present in the aquatic environment (Cavas & Konen 2008; Ergene et al. 2007). The presence of micronuclei may be caused by unrepaired or badly repaired DNA breaks (Heddle et al. 1991; Islam et al. 2019; Khan et al. 2018; Sadiqul et al. 2016). Similar morphological alterations of the micronucleus in erythrocytes have been reported in rainbow trout, *Oncorhynchus mykiss*, exposed to PAH compounds (Brinkmann et al. 2014). Taking into account these earlier findings, our data for *L. calcarifer* suggest that the appearance of erythrocytic nuclear abnormalities was the result of environmental distress that led to reductions in respirational functions, as proposed by Carrasco, Tilbury and Myers (1990).

We also observed three different types of leucocytes in the peripheral blood of *L. calcarifer*: neutrophils, monocytes, and lymphocytes. Increased numbers of leucocytes have been observed in fish collected from a polluted area (Nussey et al. 1995) and from unfavorable environmental conditions (da Silva Correa et al. 2017). The results of the present study are consistent with these earlier reports. Neutrophils with a banded or multi-lobed nucleus (bilobed or trilobed) (Figures 2(F)-3(G)) and monocytes with light basophilic nucleoplasm (Figure 2(H)-2(I)) were observed throughout the lymphocytes (Figure 2(H), 2(J)), as similarly observed in some teleosts (Singkhanan et al. 2019) and in *Danio rerio*, *Cyprinus carpio carpio*, and *Oreochromis niloticus* (Megarani et al. 2020).

We found that lymphocytes were the most abundant type of leucocyte and their numbers increased considerably during the field observation, which was apparent during the post-exposure period (Table 1). However, little information is available on lymphocytes for monitoring purposes. Murtha, Qi and Keller (2003) found that the lymphocyte proportion reached 71-92 %, but the reason was unclear. In a comparative immunology study, lymphocytes produced cytokines and responded to cellular antigens (Nakanishi et al. 2011; Wang & Secombes 2013). The formation of lymphocytes might have posed a physiological stress for the fish in our study. Monocytes were also observed and, although the role of fish monocytes is still unclear, infection, inflammation and tissue injury could all be involved (Clauss, Dove & Arnold 2008; Okabe & Medzhitov 2016). Therefore, the observation of monocytes in *L. calcarifer* might be attributable to stress caused by various unfavorable health factors (da Silva Correa et al. 2017).

## Conclusions

This hematological study observed and characterized blood cells of *L. calcarifer* that were reared in waters with a history of pollution off Sichang Island in the Gulf of Thailand. Erythrocytic nuclear abnormalities, especially the presence of micronuclei, were observed. Our findings indicate early manifestations of environmental risk that impaired the health of *L. calcarifer*. Long-term biomonitoring of the water quality and the implementation of environmental regulations need to be addressed to protect this marine environment and the well-being of all life in the region.

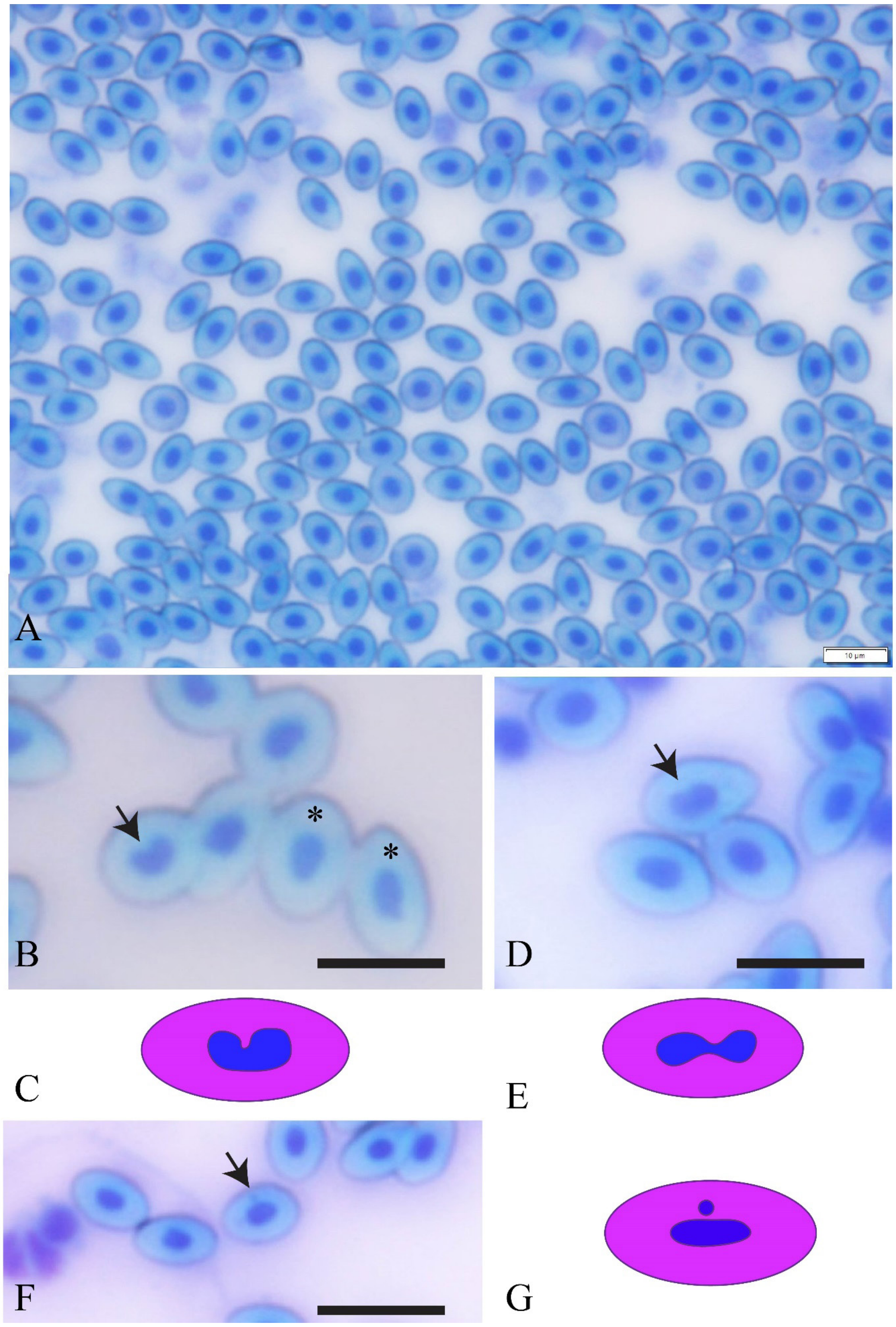


FIGURE 1. Blood was sampled from Asian seabass (*Lates calcarifer*) exposed to PAH-polluted seawater to investigate the numbers of normal erythrocytes and erythrocytes with nuclear abnormalities. A: an overall view of a blood smear slide showing abundant erythrocytes. B-C: normal erythrocytes (asterisks) and erythrocytes with kidney-shaped abnormal nuclei. D-E: erythrocytes with segmented abnormal nuclei. F-G: micronucleus

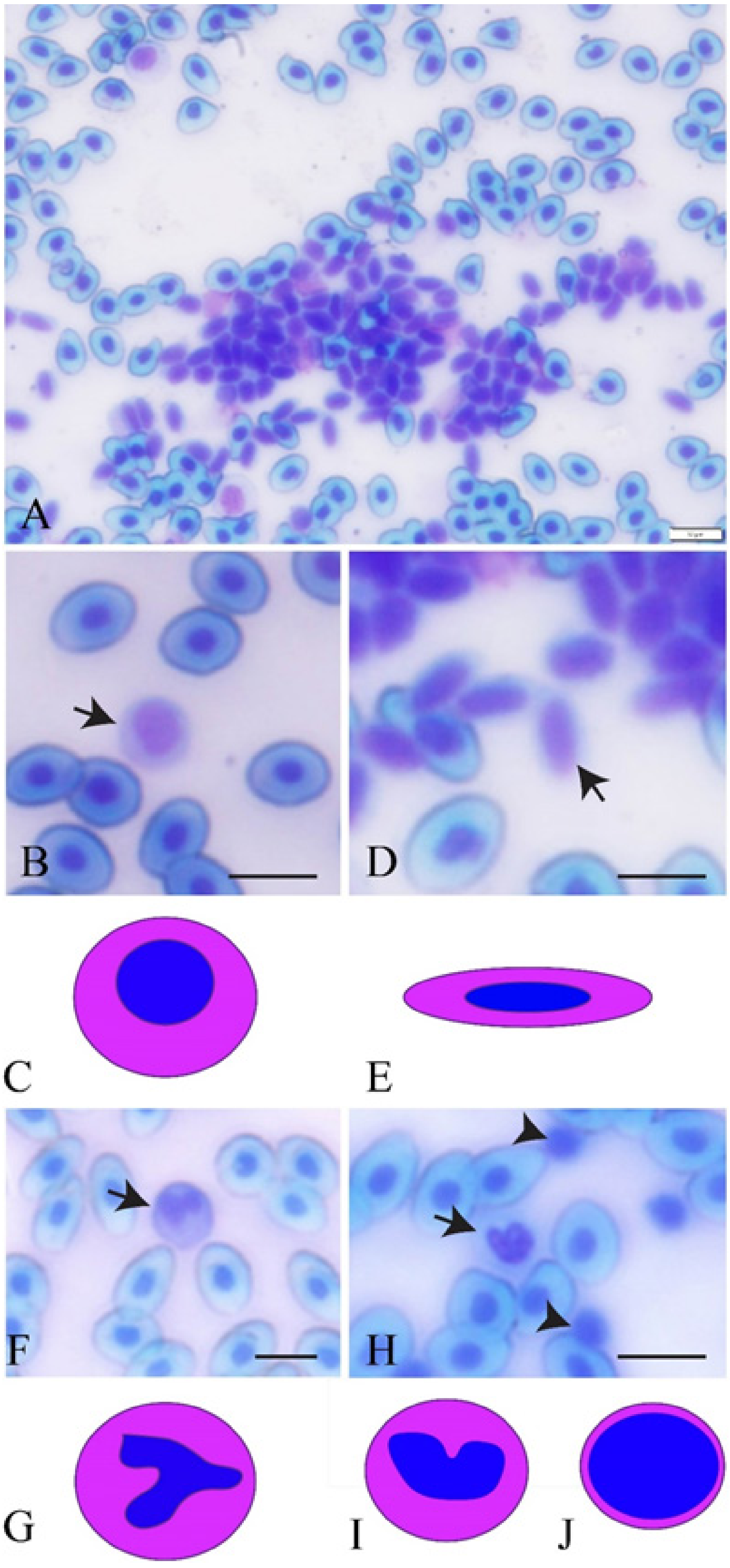


FIGURE 2. Blood was sampled from Asian seabass (*Lates calcarifer*) exposed to PAH-polluted seawater to build a leucocyte profile and investigate the presence of thrombocytes. A: the appearance of leucocytes in a blood smear slide. B-C: an azurophil. D-E: a thrombocyte. F-G: a neutrophil. H: a monocyte (I) and lymphocytes (J)

TABLE 1. Blood cell profile and erythrocytic nuclear abnormalities of Asian seabass (*Lates calcarifer*) exposed to PAH-polluted seawater in the Gulf of Thailand (Mean±SD) at 30, 60 and 90-day post-exposure

| Month | Azurophil | Thrombocyte | Nuclear abnormalities of erythrocytes | | | | Leucocytes | | |
|---|---|---|---|---|---|---|---|---|---|
| | | | Segmented shape | Kidney shape | Notched shape | Micronucleus | Neutrophil | Lymphocyte | Monocyte |
| 1 | 1.7±1.71 | 1.25±0.43 | 0.16±0.01 | 0.96±0.75 | 0.16±0.46 | 0 | 0.067±0.01 | 1.86±1.34** | 0.26±0.77 |
| 2 | 3.03±0.0*** | 3.41±0.86 | 0.06±0.01 | 0.77±0.33 | 0.033±0.01 | 0 | 0.067±0.01 | 4.13±1.64 | 0.06±0.01 |
| 3 | 1.56±4.90*** | 1.90±0.61 | 0 | 0.1±0.57 | 0.033±0.01 | 0.03±0.01 | 0 | 6.9±3.12** | 0 |

The symbol ** indicates a significant difference (p<0.01); and *** indicates a significant difference (p<0.001) when the data are compared with other groups

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