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Dear Esteemed Readers, Authors, and Colleagues,

I hope this letter finds you in good health and high spirits. It is my distinct pleasure to address you as the Editor-in-Chief of Integrative Omics and Applied Biotechnology (IIOAB) Journal, a multidisciplinary scientific journal that has always placed a profound emphasis on nurturing the involvement of young scientists and championing the significance of an interdisciplinary approach.

At Integrative Omics and Applied Biotechnology (IIOAB) Journal, we firmly believe in the transformative power of science and innovation, and we recognize that it is the vigor and enthusiasm of young minds that often drive the most groundbreaking discoveries. We actively encourage students, early-career researchers, and scientists to submit their work and engage in meaningful discourse within the pages of our journal. We take pride in providing a platform for these emerging researchers to share their novel ideas and findings with the broader scientific community.

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Our journal continues to serve as a hub for knowledge exchange, providing a platform for researchers from various fields to come together and share their insights, experiences, and research outcomes. The collaborative spirit within our community is truly inspiring, and I am immensely proud of the role that IIOAB journal plays in fostering such partnerships.

As we move forward, I encourage each and every one of you to continue supporting our mission. Whether you are a seasoned researcher, a young scientist embarking on your career, or a reader with a thirst for knowledge, your involvement in our journal is invaluable. By working together and embracing interdisciplinary perspectives, we can address the most pressing challenges facing humanity, from climate change and public health to technological advancements and social issues.

I would like to extend my gratitude to our authors, reviewers, editorial board members, and readers for their unwavering support. Your dedication is what makes IIOAB Journal the thriving scientific community it is today. Together, we will continue to explore the frontiers of knowledge and pioneer new approaches to solving the world's most complex problems.

Thank you for being a part of our journey, and for your commitment to advancing science through the pages of IIOAB Journal.



Yours sincerely,

Vasco Azevedo

Vasco Azevedo, Editor-in-Chief
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ARTICLE

COGNITIVE RADIO: NEXT FACE OF COMMUNICATION

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ABSTRACT

Cognitive Radio is the new technology in the field of communication. It is the demand of the modern era. Due to the high increase of the number of users, the call congestion or the call drops are big problem today. CR is developed as a permanent solution of these issues. Without increasing the resources, Worldwide, it is taken as a ideal and dynamic approach for improving the exploitation of a precious natural resource: the radio electromagnetic spectrum it is also enhancing the quality of services and efficiency. This technology is dedicated to use the available resources optimally and provides the solutions. This Spectrum deployment can be enhanced extensively by making it possible for a secondary user (who is not being serviced) to access the unoccupied/unused spectrum hole at any instant of time by the primary user at the right location and the time in question. This paper presents the brief introduction of the concept; types and working style of CR. A few enlightenments are also spread over the development the CR.

INTRODUCTION

Cognitive radio (CR) is a 5G machinery that comes beneath IEEE 802.22 WRAN or telecom Regional Area complex levels. Nowadays, it is undergoing through rapid development due to the ability of solving several issues, which influence and in terns degrade today's telecom network systems. The major aim for the introducing cognitive radio networks is to enhance the quality of communication. Though it is not a vital renowned technology, therefore it is necessary to make its knowledge generalized [1]. Again, due to it is a newly established techniques, there are various aspects which need to be optimized or discovered by the researchers worldwide in order to improve the services and make CR technology stable. Cognitive Radio is arranged and designed in order to use the best and available telecom channels in its proximity to ignore call drops and overcrowding. This radio spontaneously discover available, accessible and convenient channels in telecom spectrum then according to the requirement of the service it conveniently changes the necessary parameters for further synchronous telecom transmissions in a provided band of spectrums at any position. This procedure is a strong kind of spectrum authority.

CR WORKING

According to the operator's orders the cognitive engine efficiently construct radio network structure limits based on some specific parameters such as waveform, protocol, operating frequency, networking etc. These parameters acted as an independent unit in the communication surrounding and provide the required information of the network, systems and other cognitive radios available. A cognitive radio supervises its own performance continuously based on the achieved output. Then CR utilize this data to discover or detect the RF surrounding, channel conditions, link performance, etc. in order to modify the settings to optimized the quality of services at its best level in terms of user necessity, operational limitations, and regulative restrictions.

A few smartest radio plans merge the telecom network systems forcefully and changes the path of the messages among the nodes with the help of cooperative diversity technique. The frequency band of the messages is continuously flipped by the cognitive radio and also the protocols of the transmission are dynamically changed by using software defined radio to prevent the hand-off/overcrowded situations.

In other words, it is observed that the CR must possess two major characteristics for proper working i.e., [2]

Cognitive capability:- This measures the degree of identification of the fully or partially unused spectrum at very precise time or exact position during real-time interaction with the radio environment. It also defined the spectrum selecting, sharing and exploiting ability of the CR [x]. Key factors on which the cognitive capability depends are:

- Spectrum Identification
- Spectrum Analysis
- Spectrum Decision

Re-configurability: This defined the capability of CR for selecting the best spectrum band and proper working parameters and flexibility of reconfiguring of the selected one if necessary within at instance [x]. The major parameters, on which the reconfigurability follows are:

KEY WORDS

Unused Channel, White Space, Frequency Spectrum, Call Congestions, Call Drops

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- a. Transmission Power
- b. Operating Frequency
- c. Communication Technology
- d. Modulation Techniques

HISTORY OF CR

The idea of CR (cognitive radio) was initially introduced by Joseph Mitola III in a conference organized at KTH in the year 1998. It was a novel proposal in the field wireless communications, which was described by Mitola as:

“The point in which wireless personal digital assistance and the related networks are sufficiently, computationally intelligent about the radio resources and related computer to computer communication to detect user communications needs as a function of use contexts and to provide radio resources and wireless services most appropriate to those needs ”

Cognitive radio should be developed as completely re-configurable telecom transmitters, which need to be accommodated within the transmission framework of the system and can act according to the requirement of the service/user. Usually regulative parameters are made for analog model and there is no such rules developed for the CR. Again, these bodies from worldwide reported not proper/unsufficient usage of the frequency spectrum [1]. As an example the cellular network facing heavy load whereas another frequency bands dedicated to other applications are free most of the time. According to the various studies and researches, it is observed that the load of the frequency spectrum is highly dependent on the time and place, which in terms shows the insufficient consumption of the channels. The permanent allotment of the frequency spectrum to any application is prevents from the interference. But due to the limited frequency band and overcrowding situation it is necessary that the allocation must be dynamically. Now the regulatory bodies are forced to arrange a system, which can assign/allow the unlicensed users to use the licensed bands without creating any types of interference. This need leads to the path towards the cognitive radio technique. In order to establish the concept of cognitive radio, IEEE first set the standards in the year of 2011. This standard mainly focused on, to spread the awareness of CR concept through the spectrum sensing and geo-location. These parameters first combined with the database of the licensed transmitter and identify the accessible channels. Now, these standards are designed in such a way that it can use or reuse the available frequencies for a time slot in desired location according to the requirement of the service and without disturbing that licensed user. However CR is not capable to use the same frequency band for all the time.

HISTORY OF CR

There are two major types of cognitive radios:

Mitola radio (complete cognitive radio):- In this arrangement, the wireless node is advised to observe all the necessary parameters [3].

Spectrum sensing:- In this arrangement only the frequency spectrum is advised to observe. Other CRs depends on the following parts of the spectrum for acquiring the channels as per the need:

- Licensed Band Cognitive Radio: It has the capability of accessing the licensed user bands. It works under WRAN and usages the unused television channel frequencies named white spaces.
- Un-licensed Band Cognitive Radio: it can only access the unlicensed radio frequency spectrum. The system under which it is working is defined by the IEEE 802.15 task group. It is mainly focus on the co-existence of two different specifications i.e. IEEE 802.11 and Bluetooth connectivity.
- Spectrum Mobility: It uses the mobility feature as its major advantage. The CR flipped its operational frequency consistently. Its major focus is to use the spectrum dynamically and operates at the best frequency in order to continue seamless communication during hand-off/shifting radio spectrum situations.
- Spectrum Sharing: It is mainly operating by sharing the licensed spectrum bands. The issue of interference is maintaining low or under a threshold value by limiting the transmitting power of CR [4].
- Sensing based Spectrum Sharing: In this scheme, at first the CR is assigned with the licensed spectrum in order to estimate the situation of the licensed users present at that frequency band. After estimating the condition, the CR selects its transmission plan. If the licensed bands are available, cognitive network uses the bands, however if it is not free the network shares these spectrum bands with the users and maintain their transmission power low in order to avoid the interference [5].
- Database enabled Spectrum Sharing: In this scheme, at first the demand is placed for accessing the white space database. On the basis of several algorithms few mathematical models are developed under the local regulatory bodies for the prediction of the utilization of the spectrum bands. After processing these criteria, the decision of allocation or denied is made. The risk

assessments, crowding condition and geo area are also considered before taking the decision [6].

CONCEPT BEHIND TECHNOLOGY

Initially, the concept of CR is considered as the expansion of software defined radio. Several research works are on-going, where the main focus is to analyse the spectrum sensing feature of CR. The major obstacle in this is to design the high quality algorithm or protocols for sensing the data. The detection of the availability of signal can't be assured by a conventional energy analyzer. Therefore the need of more precise techniques for sensing the frequency spectrum and fetching information about the regular swapping of data among nodes are highly required. As the enhancement in cooperative sensing nodes, the chance of failure or false detection reduces. Hence, the concept behind the development of the CR is highly based on cognitive networks which can maintain or organize the grid of cognitive radios smartly. The [Table 1] below represents some of the major previous works done by the researchers from worldwide [7].

Table 1: Some Major Previous Work along with their Findings and Loopholes

Sl. No.	Author Name	Objective	Findings	Loopholes
1	S.A. Jafar et. al. [8]	To find out the overall potential of CR	The status of the spectrum is identified by the spectrum sensing. It also senses the activity of licensed users by sensing the target frequency band.	Not implemented yet
2	Shi Gaungqiang et. al. [9]	To share the cooperative spectrum between multiple PU's and SU's	It has confliction between the PU and SU for exchanging the resources in terms of relay power and time allocation	The design is not completely developed for PU's
3	Gautam Ghosh et. al. [10]	To implement the CR architecture, which is enabled with the artificial intelligence	For improving the communication efficiency, the opportunistic access of the available channels are allowed by the dynamic spectrum to users	Security issues
4	Ian F. Akyildiz et. al. [11]	To study on xG network for developing and enhancing the standards and resolving the issues of wireless network CR	CR is able to identified and accessed the best available channels using dynamic spectrum techniques	It faces the transport and cross layer challenges
5	M. Sisk Behaei et. al. [12]	To study about the usage of full duplex and half duplex in order to enhance the CR efficiency	Primary channels are sensed by the half duplex radio	Some channels are dedicated as full duplex in network
6	Bijal K. Jariwala et. al. [13]	To afford more wireless devices with additional frequency and band reliability	CR is highly reliable communication mode due to having capability of changing operative parameters according to the requirement of users, services and environment	Limited spectrum
7	Shewangi et. al. [14]	To study about the re-configurability of SDR	It is possible to make CR as re-configurable SDR due to its ability of detecting changes in environment and acting accordingly	Unwanted disturbance due to the CR devices are not detected by Primary users in CSMA (carrier sense multiple access) scheme
8	G. Manikandan et. al. [15]	To study about the spectrum sensing ability of cooperative and non-cooperative parameters in order to enhance the efficiency of CR	CR is highly reliable communication mode and one of the ideal approach for proper utilization of natural resources	False interpretation due to uncertain strength of received signals

SPECTRUM SENSING

As The major motivation behind the cognitive radio is the efficient utilization of spectrum. To process in this direction, CR proceeds through following steps:

- Sensing of available spectrum
- Management of spectrum and hand off
- Sharing and allocation of spectrum
- Spectrum mobility

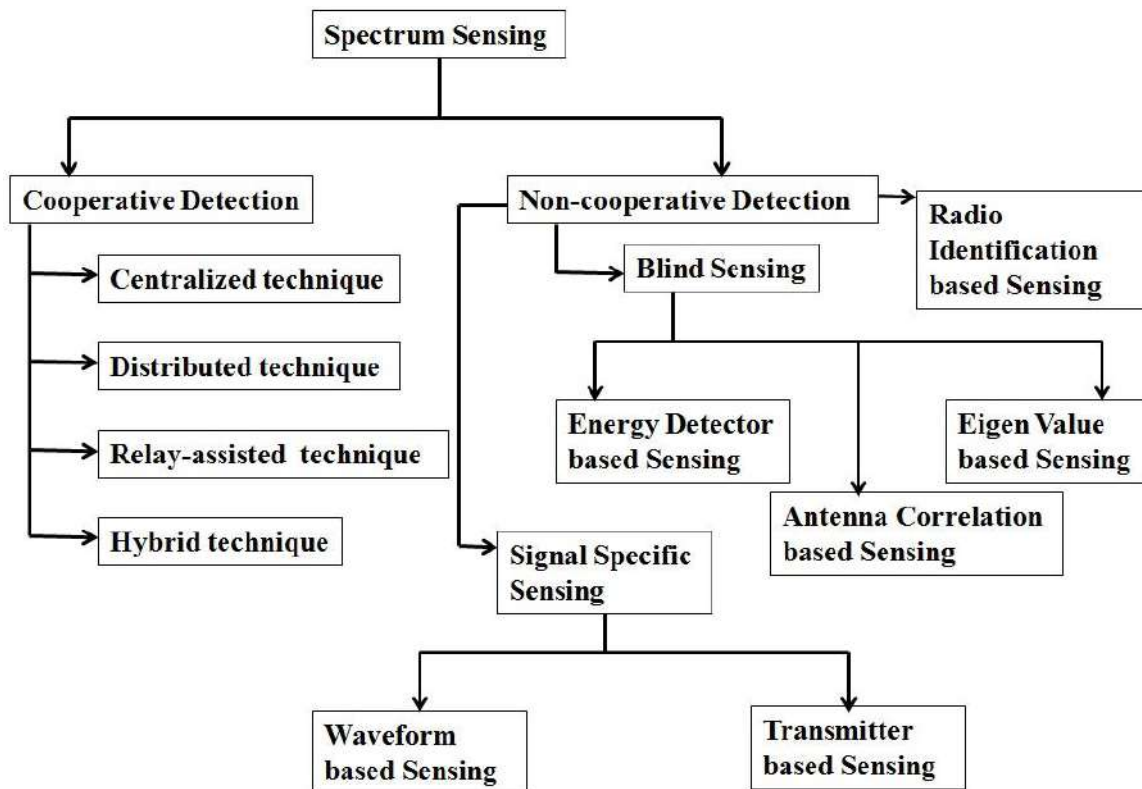


Fig. 1: Various Spectrum Sensing Techniques

As discussed, at first CR needs to sense the spectrum efficiently, which is the key function for the working of CR. There are mainly two different types of approach namely Cooperative Detection and Non-cooperative detection. The Spectrum Sensing techniques are represented in [Fig. 1].

Cooperative detection:- In this method, the secondary user collects and transmits all the information about the availability of unoccupied channels of the spectrum. This information is then transmitted to the central coordinator, which is responsible for the selecting the appropriate one according to the required parameters such as time, situation etc. and revert its decision to the secondary user [16]. The CR systems also share this information among one another in order to enhance the efficiency of the system [17].

There are mainly three different types of techniques used for the cooperative detection.

- Centralized technique:-** In this technique, there is a dedicated central processor, which controls entire network. This processor is responsible for the identification, collection and analyzes the spectrum in order to determine the available channels and share this information with other CR networks. To reduce the fading effect, the central processor can also control the cognitive radio traffic [18].
- Distributed technique:-** In this technique, nodes are capable to analyze the free channels and share this information with the other existing nodes. This technique reduces the cost due to less infrastructure requirement in terms of central processor but it needed large storage and computation ability for efficient processing [18].
- Relay-assisted technique:-** In this technique, channels are sensed and the information is transmitted to the target node through several intermediate hops. These working hops are relays. This structure is also known as multi-hop cooperative structure sensing [16].

- D. Hybrid technique:- In this technique, the decentralized scheme is used for sharing information. All the channels identification are done by users themselves and the acquired channels are immediately vacate when the primary user needs for it without sharing this information to other nodes. Using this technique saves the time, although a dedicated separate hardware unit is required for the efficient processing, which increase the cost factor [17].

Non-cooperative detection:- In this method, all the measurement, acquisition and analysis regarding the spectrum occupancy is done by the radios itself (individually). These local radios are act as an autonomous body and carry all the information about its spectrum.

Broadly three different approaches are dedicated to this detection technique.

- A. Blind Sensing:- This technique is also based on a devoted node named as Fusion Center, which collects and analyzes the information getting from the sensing nodes present in the entire network in order to determine the frequency, which is used as a key parameter [16].
- B. In blind sensing model, there are several schemes such as:
- Energy Detector based sensing:- A simple method that is implemented using Fourier transform algorithms. In this technique, the information about the primary user is very limited. Again, here the threshold value is highly depends on signal to noise ratio (SNR) [19].
 - Eigen Value based sensing:- In this method, the threshold value is one of the quantized value of the ratio of maximum to minimum eigen value. This eigen values are determined using the covariance matrix of the received signal [20, 21].
 - Antenna Correlation based sensing:- In this method, the threshold value is chosen by exploiting antenna correlation based detector from time domain to space domain [22].
- C. Signal Specific sensing:- This technique is based on the prior information about the primary user [16].
- D. Various models are used under this sensing technique:
- Waveform based Sensing:- This method is also known as coherent sensing technique, which is only applicable to a system when the signal pattern of the system is already identified [23-25].
 - Transmitter based Sensing:- This technique is based on the weakest signal, which is transmitted by the primary user. Again the method of energy based sensing and correlation based sensing is used to proceed further in order to make an efficient network [16].
- E. Radio Identification based Sensing:- This model concentrate on the level of the interference at the receiver end. This measured interference is named as interference temperature [16].

CONCLUSION

CR is highly intelligent technology and an important breakthrough achieved by the human being in the field of communication. It seems to be a solution of call congestions and call drops without arranging any extra resources. This technology ensures that the resources are completely and optimally used for the services. The unused channels are allocated and work efficiently to fulfill the requirements and if the call comes from their dedicated users, with the help of CR techniques, the services shift to the other available resources and the previous channel again assigned back to their original defined duty. In this way the CR enhance the potential of the spectrum without increasing the natural resources and make a significant difference to wireless communications, hence, referred as “disruptive, but unobtrusive technology”. Both the licensed and unlicensed users can be benefitted with the CR. This technology is the remedy of the channel deficiency and next face of communication.

CONFLICT OF INTEREST

There is no conflict of interest.

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None.

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ARTICLE

A COMPARATIVE REVIEW OF NH₃-H₂O AND H₂O-LiBr BASED VAPOR ABSORPTION REFRIGERATION SYSTEMS

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ABSTRACT

The vapor absorption refrigeration (VAR) system is gaining importance due to its ability to reduce energy wastage and utilize available energy resources efficiently. This system also provides an adequate substitute for traditional refrigerants such as Hydrochlorofluorocarbons (HCFCs) and Chloro-fluorocarbons (CFCs). However, ozone depletion and global warming problem might be reduced using this system. The primarily used environment-friendly VARS is a mixture of NH₃-H₂O and LiBr-H₂O. The working principle of the VARS is heating and pressure difference during evaporation and condensation process in a reverse heat engine. In this article, various parameters which can affect the efficiency of NH₃-H₂O and LiBr-H₂O VARS system are reviewed and compared. From the review, it can be concluded that the COP of LiBr-H₂O based system was always better than NH₃-H₂O based VAR system. Also, the COP of multiple effect VAR system was higher than the single effect VAR system.

INTRODUCTION

KEY WORDS
Vapor absorption refrigeration system, NH₃-H₂O, H₂O-LiBr, refrigerant, working fluid

The process of heat flow from the low-temperature system to high-temperature one by the aid of some external means is referred as refrigeration. There are ample applications based on this process such as refrigerators, freezers, cryogenics system, air conditioner etc. At present, it has a substantial impact on the lifestyle of the human race. Absorption refrigeration is a process in which some heating source is always required to provide necessary cooling comfort. The heating source might be direct or indirect heating type powered by the energy extracted from solar, gasoline or other non-conventional energy resources. The essential property of the refrigerant used for the refrigeration system is an extremely low boiling point which must be less than -18 °C. In case of an absorption refrigeration system, only heat input is required for converting vapors to liquid. Therefore, the only moving part in this type of system is refrigerant itself. The refrigerants used in this type of system are NH₃ and H₂O mostly. The vapor absorption refrigeration (VAR) system primarily utilizes low-grade thermal energy such as waste heat or solar power for running the cycle. Additionally, the working fluids employed in this system is natural and does not harm the environment in any capacity, unlike traditional refrigerants [1]. Therefore, the vapor absorption refrigeration system is eco-friendly and providing a similar level of cooling efficiency as produced by the compression refrigeration system. A regular depletion of the ozone layer has grown concern for environmental protection worldwide [2]. The use of hydrocarbons as a refrigerant in the various refrigeration system had a significant contribution to global warming. Some commonly used hydrocarbons as a refrigerant such as CFC-12, HCFC-22, HFC-134a, HFC-161, R502, R404A, R507 etc. have relatively high ozone depletion potential [3, 4]. Moreover, research on new processes working on natural refrigerant has been intensified recently. The focus was also shifted from traditional to renewable energy resources and need to have been grown to utilize the low-grade energy for the refrigeration purpose. Moreover, research on natural refrigerant-based refrigeration system has been intensified recently. The focus has also been shifted from traditional to renewable energy resources. The process optimization is kept on improving for the utilization of low-grade energy which is assisting in higher energy efficiency [1, 2, 5].

In this article, a comprehensive review of the performance of commonly used natural refrigerants i.e. NH₃ and H₂O in VAR system is summarized. The effect of several parameters on the performance of the VAR system is also reviewed. This paper also compiles various procedures used to enhance the coefficient of performance (COP) of NH₃-H₂O and LiBr-H₂O absorption cycle.

VAPOR ABSORPTION REFRIGERATION SYSTEM

The working principle of the vapor absorption refrigeration system is the existence of a necessary pressure gradient between the vaporization and condensation processes. The working fluid released heat to the environment when condensed under high pressure. Whereas, in the case of evaporation, the heat is absorbed from the medium or surrounding and vaporized under low pressure. The cooling effect is produced during the evaporation process. This technique is adopted widely for the utilization of available conventional and unconventional energy resources efficiently [1]. It assisted in terms of minimizing the energy wastage and replacing the traditional refrigerant (HCFCs CFCs etc.) with more environmental friendly working fluids. Using this technique not only prevented ozone layer depletion but also reduced greenhouse warming. The NH₃-H₂O working fluid is the only environment-friendly mixture utilized currently for refrigeration purpose. The applications of VAR system is divided into two ranges i.e., above and below 10 °C. The commonly used working refrigerant-absorbent fluid pair of VAR systems for above and below

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10 °C temperature range applications are H₂O-LiBr and NH₃-H₂O, respectively. The H₂O-LiBr fluid pair could not be used for extremely low-temperature applications owing to the crystallization tendency of LiBr. At extremely low temperatures the miscibility of LiBr and H₂O is poor and phase separation will take place. The selection of absorbent for VAR system was done on the basis of their ionic nature. The fluid with ionic nature has good solubility in different natural refrigerants along with low volatility. Besides the advantages, the high circulation ratio and viscosity of such type of fluids require high pumping power consequently poor absorber efficiency. This problem might overcome if water is used as a co-solvent. Various works have explained theoretically and experimentally the significance and viability of different working fluid operated VAR system processes in terms of its performance and cost-effectiveness. The work explained the process and performance optimization of NH₃-H₂O and H₂O-LiBr working fluid pairs are summarized in forthcoming sections [2].

NH₃-H₂O VAR SYSTEM

NH₃-H₂O refrigerant-absorbent pair is frequently used in an absorption refrigeration system from the starting onwards. This pair is stable in a broad range of working temperature and pressure. NH₃ possess high latent heat of vaporization, and extremely low freezing temperature (-77 °C) resulted in an improved performance at low-temperature applications [6]. A rectifier is required in the cycle owing to the volatile nature of both the fluids to fetch the maximum performance from the system. Some disadvantages of the pair are colossal pressure, toxicity, and corrosion behavior towards Cu and its alloys [7]. A simple block diagram of a simple NH₃-H₂O VAR system is shown in [Fig. 1].

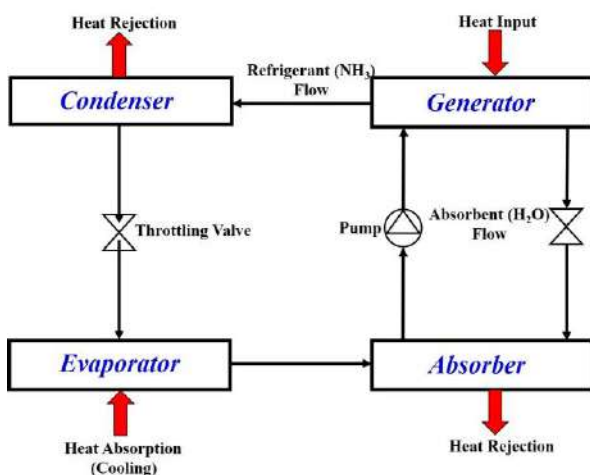


Fig. 1: Block diagram of NH₃-H₂O refrigeration system [10].

Ramesh et al. [8] investigated the performance of NH₃-H₂O vapor absorption system with shell and helical coil type of solution heat exchanger. They observed that the tube side heat transfer coefficient was higher than the shell side. Also, the Nusselt number of tube side was four times higher than the shell side. Lee et al. [9] added Al₂O₃ nanoparticles and carbon nanotubes in pool type NH₃-H₂O VAR system and studied the performance. They noticed 0.02 vol% Al₂O₃ nanoparticles addition exhibited optimum performance. Raghuvanshi and Maheshwar [10] studied the performance of NH₃-H₂O VAR System using the first law of thermodynamics. The COP decreases with higher generator, condenser and absorber temperatures whereas; it increases if the effectiveness of heat exchanger increases. Du et al. [11] worked on a prototype of the air-cooled two-stage NH₃-H₂O solar VAR system for residential use. The prototype performed smoothly and steadily with a COP range of 0.18-0.25. Kim et al. [12] examined the effect of surfactants such as 2-ethyl-1-hexanol, n-octanol, and 2-octanol on the bubble type NH₃-H₂O vapor absorption system. The absorption performance was improved by 4.81 times after the addition. Kang and Kashiwagi [13] quantified the impact of n-octanol (heat transfer additive) addition on Marangoni convection in NH₃-H₂O absorption process. They observed that the increased heat transfer additive concentration improved the driving potential of absorption resulted in high Marangoni convection. Therefore, the absorption performance of NH₃-H₂O VAR system improved significantly. Fernández-Seara and Vázquezan [14] obtained improved COP of NH₃-H₂O VAR system with an inefficient solution heat exchanger and higher pressure drop between evaporator and absorber. Sieres and Fernández-Seara [15] judged the effect of distillation column in small capacity NH₃-H₂O VAR system using numerical analysis. The use of stripping section in the distillation column improved the COP values up to an absolute limit thereafter no improvement was proclaimed. Khaliq and Kumar [16] compared the exergy destruction for NH₃-H₂O and LiBr-H₂O working fluid in a single effect VAR cycle. Considerably high exergy destruction was inscribed for NH₃-H₂O than the LiBr-H₂O solution. The exergy loss mainly occurred in the generator and absorber. With the increase of absorber temperature, exergetic efficiency improved notably. Rogdakis and Antonopoulos [17] proposed a new work producing cycle for NH₃-H₂O VAR system and compared its

performance with the Rankine cycle. For all the conditions, the proposed cycle exhibited better efficiency than the Rankine cycle. Jawahar and Saravanan [18] examined the performance of an air-cooled modified NH_3 based generator absorber heat exchange (GAX) absorption cooling system. The optimum cooling capacity was reported when the generator and evaporator temperatures were 120°C and 2°C , respectively.

H_2O -LiBr VAR SYSTEM

H_2O -LiBr refrigerant-absorbent pair was first used in a VAR system in around 1930. A schematic diagram of a simple H_2O -LiBr VAR system is exhibited in [Fig. 2]. There is no need of rectifier in this system as LiBr is non-volatile and H_2O owns a high heat of vaporization. The use of H_2O , as a refrigerant, limits its application above 0°C temperature and require vacuum for smooth functioning. The presence of LiBr in an excessive amount makes the solution more susceptible to crystallization. This problem might get solved if the salt such as ZnBr_2 , ZnCl_2 etc. is added to the working fluid. The COP of this system is always better than the NH_3 - H_2O couple. This system is only used to generate air conditioning effect [19, 20]. Saravanan and Maiya [21] did the performance analysis of H_2O -LiBr VAR system operated by the bubble pump. They recorded the highest COP of 0.5 when the boiler temperature was 85°C and absorber and condenser temperatures were more than 40°C . Asdrubali and Grignaffini [22] evaluated the performances of H_2O -LiBr VAR system in various circumstances. They found acceptable efficiency of the machine when the input temperature was as low as 65 - 70°C . This result indicated that the same device could be driven by solar power. Kang et al. [23] studied the heat and mass transfer rate of H_2O -LiBr falling film absorption process with the addition of Fe or CNT nanoparticles. The mass transfer rate was higher with CNT nanoparticles added working fluid resulted in improved absorption performance of the system. Arora and Kaushik [24] developed a computational model for performance analysis of single and double effect LiBr- H_2O VAR systems. Although the COP and exergetic efficiency increased with the increase of generator temperature, the higher absorber, condenser and evaporator temperatures decreased the same in both the system. The highest amount of irreversibility was recorded in the absorber. Kim et al. [25] did the simulation of the compressor-assisted triple-effect H_2O -LiBr absorption cooling cycles. The corrosion is the main problem with LiBr based triple-effect absorption cooling system. They proposed a new four compressor-assisted H_2O -LiBr cooling system and observed no danger of corrosion with LiBr working fluid. The 1.5-effect H_2O -LiBr cycle based absorption system exhibiting increased sensitivity towards the change of absorption temperature, consequently the generation temperature will alter [26]. Domínguez-Inzunza et al. [27] compared the performance of H_2O -LiBr operated single, half, and double effect VAR systems. The minimum and maximum COP was observed with half and double effect VAR system, respectively. The maximum COP of complex designed double effect VAR system was 1.48.

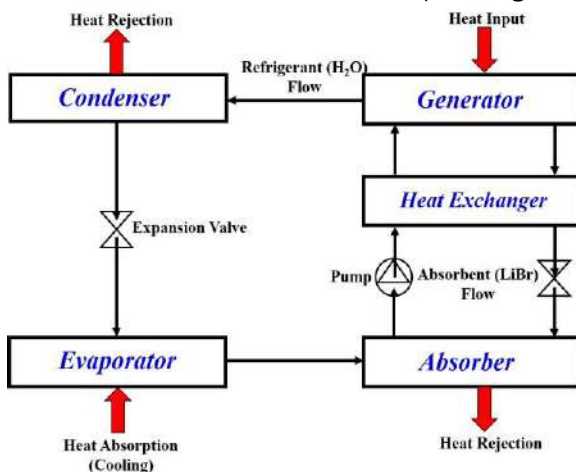


Fig. 2: Block diagram of H_2O -LiBr refrigeration system [24].

VARIATION IN COP

The value of a coefficient of performance (COP) of a refrigeration and air conditioning unit is determined by the ratio of heat absorbing ability of evaporator to the total power supply to the unit. The COP is inversely proportional to the operating cost of the unit. Therefore, a higher COP translated into the lower operating cost. Several efforts have been made to improve the COPs of the compression or absorption refrigeration systems. The COP is varied with the choice of a refrigerant-absorbent pair, single stage or multiple stage operation, generator temperature, absorber temperature, con-denser temperature and various operating pressures. A list of COPs reported in literature considering multiple parameters are listed in [Table 1]. It was observed from the table that H_2O -LiBr couple is providing better COP than NH_3 - H_2O couple. The multi-stage operation exhibited improved COP than the single-stage operation. It was even touching the value of 1.9 in case of triple effect cycle for predefined generator, absorber and condenser temperature.

Table 1: The COP values at different parameters and working pairs

S. No.	Working Fluid	Operation Type	Generator Temp (°C)	Evaporator Temp (°C)	COP	Cooling Capacity (kW)	Year	Ref
1	NH ₃ -H ₂ O	Single effect	100	-5	0.6160		1998	[28]
	NH ₃ -LiNO ₃	Single effect	100	-5	0.6247			
	NH ₃ -NaSCN	Single effect	100	-5	0.6390			
2	R1234yf/[hmim][Tf2N]	Single effect	90	5	0.363	100	2017	[29]
	R1234ze(E)/[hmim][Tf2N]	Single effect	90	5	0.426	100		
	H ₂ O-LiBr	Single effect	90	5	0.805	100		
	NH ₃ -H ₂ O	Single effect	90	5	0.737	100		
3	NH ₃ -H ₂ O	Air-cooled two-stage	85	8	0.18-0.25	2	2012	[30]
4	H ₂ O-LiBr	Single effect	60-190	4-10	0.73-0.79	300	2009	[31]
	H ₂ O-LiBr	Double effect	60-190	4-10	1.22-1.42	300		
5	NH ₃ -H ₂ O	Single effect	100	10	0.646	1112	2005	[32]
	H ₂ O-LiBr	Single effect	100	10	0.833	2502		
6	H ₂ O-LiBr	Single effect	90	5	0.770		2007	[33]
7	H ₂ O-LiBr	Single effect	87.8	7.2	0.7609		2009	[34]
	H ₂ O-LiBr	Double effect	140.6	7.2	1.26			
8	H ₂ O-LiBr	Single effect	90	5	0.76		2010	[35]
	NH ₃ -H ₂ O	Single effect	90	5	0.54			
	NH ₃ -LiNO ₃	Single effect	90	5	0.55			
9	NH ₃ -H ₂ O	Single effect	121.1	-11.2	0.47	10.51	1996	[36]
10	H ₂ O-LiBr	Double effect	85-170	2.5-10	1.32-1.39		2000	[37]
11	H ₂ O-LiBr	Single effect	60-225	4-10	0.73-0.79	300	2010	[38]
	H ₂ O-LiBr	Double effect	60-225	4-10	1.22-1.42	300		
	H ₂ O-LiBr	Triple effect	60-225	4-10	1.62-1.90	300		
12	H ₂ O-LiBr	Single effect	65-85	5	0.467-0.644	2	2007	[39]

CONCLUSION

In this article, a comparison of VAR systems based on NH₃-H₂O and H₂O-LiBr working fluid was carried out. The literature regarding the same was reviewed and summarized. NH₃-H₂O pair can be used in refrigeration as well as air conditioning applications whereas; the H₂O-LiBr pair can be used in air conditioning applications only. The problem of crystallization of absorbent and the freezing tendency of a refrigerant at extremely low temperature constrained its use in refrigeration applications. The addition of efficient heat exchanger designs and/or surfactant or heat transfer additive in working liquid enhanced the COP in NH₃-H₂O based VAR system. The COP of LiBr-H₂O based system was always better than NH₃-H₂O based VAR system. Also, the COP of multiple effect system was higher than the single effect system.

CONFLICT OF INTEREST

None

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FINANCIAL DISCLOSURE

None

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ARTICLE

CHALLENGES AND CONTROLLING STRATEGIES OF MOSQUITO

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ABSTRACT

In recent era, mosquito-borne deadly diseases are accounting approximately about 17% of all the infectious diseases. Although Malaria is the principal focus of the scientists, other deadly diseases like dengue and chikungunya are endemic in many developing countries. Though, synthetic mosquito repellents are controlling the mosquito population but they possess a lot of disadvantages to pregnant women and children. Thus, the focus has been shifted towards plant based repellents and plant derived essential oils which show efficacy with no side-effects. Research is also going on focusing the development of an anti-parasite vaccine. To this end, though, there is no licensed vaccine at present but a lot of progress is seen in this field recently. Another area of research has been focused on sterile insect techniques and transgenic mosquitoes in order to suppress the whole disease spreading female vector population. The progress in the field of molecular biology has facilitated greatly to disrupt and exploit the mosquito's life cycle. This review highlights all the approaches investigated to control mosquito-borne diseases with a fair discussion on challenges faced in this regard.

INTRODUCTION

In this highly socialized and globalized world, Mosquito borne diseases are one of the major causes of deaths every year. Mosquito-borne diseases account for about 17% of all the infectious diseases, causing more than 1 million deaths annually [1, 2, 3]. According to a survey, it is estimated that about 2.5 billion people in over 100 countries are at a risk of acquiring dengue fever alone. It is also found according to survey that malaria alone causes 600000 deaths globally every year and the saddest part is that most of them are children [1, 2]. Other mosquito-borne diseases such as dengue, West Nile virus, Japanese encephalitis etc. also cause deaths of many people every year globally. Although many of these mosquito-borne diseases are preventable if there is awareness about protective measures.

In epidemiology, a vector is any agent (person, animal or microorganism) that carries and transmits an infectious pathogen into another living organism. Mosquito-borne diseases spread through bites of infected mosquito species (vector), which mainly includes Aedes, Culex and Anopheles. These Mosquito-borne diseases are highly wide spread in few parts of the world whereas they are found in minimum numbers in certain countries of the world. They are most prominent during a particular time of the year while they are less prevalent in other times. Most vector-borne diseases exhibit a distinct seasonal pattern, which clearly suggests that they are highly sensitive to weather. Rainfall, temperature, and other weather variables also affect in many ways to both the vectors and the pathogens they transmit. Thus, the probability of transmission may or may not be increased by higher temperatures [1, 2, 4]. There are various factors which make a huge impact and bring a drastic change in the diverse spread of these vector borne-diseases in the world.

So a review of all the existing approaches towards mosquito-borne diseases is indeed necessary for people especially those who are living in the endemic areas to prevent themselves from mosquito-borne diseases. As there has been a lot of focus on different methods to exploit the vector's life cycle in preventing the disease, many improvements have been achieved by the scientists, which has been discussed in this study.

MOSQUITO LIFE CYCLE AND TRANSMISSION OF PARASITE

Despite the many differences that exist within the genus, the lifecycle of all mosquitoes is generally the same. Eggs hatch in water, where they hatch and undergo the transition to larvae. There are four aquatic larval stages. It is followed by an aquatic pupal stage before the adult arises. Adults feed on nectar and other sugar sources. Within days of emergence, adult males form mating swarms into which females fly to mate. The female must then take a blood meal before she is able to lay 50-200 eggs. Most adults can live up to 2 weeks in the field [5].

In humans, the parasites enter and multiply first in the liver cells and then they invade RBC's. In the blood, broods of parasites grow inside the RBC's and destroy them, releasing daughter parasites (merozoites) that continue the cycle by invading other RBC's and form gametocytes. The blood stage parasites are those that cause the symptoms of malaria. When certain forms of blood stage parasites (gametocytes) are picked up by a female mosquito during its blood meal, they start another different cycle of growth and multiplication inside mosquito. The male and female gametocytes form a zygote and then form an oocyst. After 10-18 days, the parasites are found in the form of sporozoites in the mosquito's salivary glands. When the female mosquito takes a blood meal on another human, the sporozoites are injected with the mosquito's saliva into human and start another human infection when they parasitize the liver cells [5].

KEY WORDS

Mosquito-borne diseases, parasites, vectors, infectious diseases.

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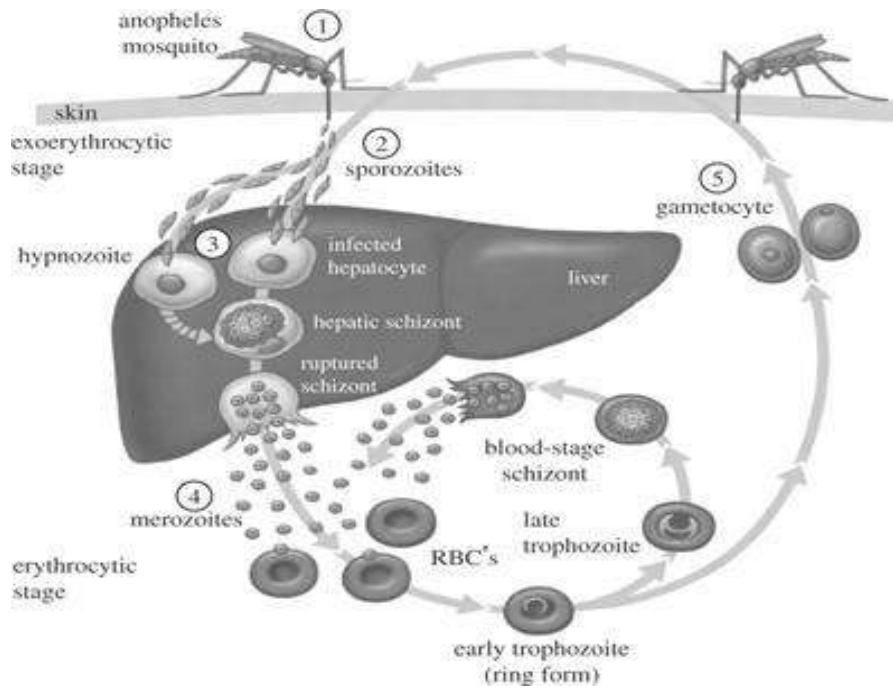


Fig. 1: Transmission and life cycle of malarial parasite, Wikimedia commons [5, 6].

METHODS USED IN CONTROLLING MOSQUITO POPULATION

Several approaches towards mosquito borne diseases have been investigated worldwide which are mainly classified into two types- genetic and non-genetic. While most of the non-genetic approaches are methods used for controlling mosquito population before the technology overtook. These approaches are highly effective in some areas and have been even employed now-a-days in many parts of the world. While genetic approaches are able to prevent and cure mosquito-borne diseases by highly cost effective interventions [3].

Non genetic approaches

Environmental control

Vector control strategies in this method involves suppressing the mosquito population through environmental modifications and manipulations like draining of stagnant water from wetlands, removal of mosquito's potential breeding habitats, and installation of meshes in homes [7, 8]. This approach has some success in controlling the mosquito population in Rome, Israel, India, Brazil, Egypt, and Zambia and is still used and recommended as an alternative approach in a few areas [9-11]

Chemical control

Early mosquito management relied on the use of Paris green (copper aceto arsenite) and petroleum by-products. It was discontinued because of their high toxicity and pollution of water sources [7, 12]. DEET (N,N-Diethyl-meta-toluamide) is a synthetic mosquito repellent that has been effective against several species of mosquitoes, however its repeated use disrupts natural biological systems. This pesticide has been reported toxic and allergic reactions in infants, children and pregnant women. It has also been found that mosquitoes gain resistance towards these synthetic repellents after a certain period of time [13-16]. Insecticide-treated bed nets alone have been regarded as an excellent tool to reduce malaria transmission in highly endemic countries, especially by reducing child mortality and morbidity [17-19].

Biological control

The western mosquito fish *Gambusia affinis* is a species of freshwater fish, also known commonly as mosquito fish. There is also an eastern mosquito fish *G. holbrooki*. The name "mosquito fish" was given because the diet of this fish sometimes consists of large numbers of mosquito larvae, relative to body size. Mosquito fishes have been introduced directly into ecosystems in many parts of the world as a bio control to lower mosquito populations which in turn negatively affected many other species in each distinct bioregion [20-23, 7].

Another type of biological approach in use against mosquitoes, is the use of entomo-pathogenic fungi. This method involves spraying mosquito's resting sites with suspension of fungal spores. Upon exposure, the

fungi readily invades and multiplies inside the mosquito, killing it within 15 days, and thus reducing the parasite's transmission intensity [24, 25]. Laboratory and field studies have identified two different fungal species, *Beauveria bassiana* and *Metarhizium anisopliae*, that are effective against *A. Gambia*. *B. thuringiensis israelensis* and *B. sphaericus* are other two environmental friendly alternatives, given that the toxins they produce are non-toxic to other species and do not persist for a long period of time in the environment [7]. Hurdles for this approach mainly includes fungal spore viability, fungal specificity, and the development of resistance in the mosquitoes [26, 27].

Essential oils and natural mosquito repellents

Essential oil obtained from plants play an important role as xenobiotics due to presence of mono and sesqui-terpenes along with other volatile carbons [26]. In this method of controlling mosquitoes, certain plants which were previously described to have mosquito repelling properties are selected and they are subjected to hydro-distillation using a Clevenger type apparatus. Then the essential oils obtained are used in arm cage studies, where the arm of human hand treated with the essential oil is kept into a cage full of mosquitoes of a particular genus. The efficiency of the oil is tested by watching the number of mosquitoes falling on the human hand at appropriate time intervals [15].

In certain experiments, leaves are dried and grounded using a mechanical blender. Then, they were extracted using polar solvents like benzene or ethyl acetate, which were again tested for mosquito repellence using arm cage studies or to check the larvicidal activity [14].

Genetic approaches

In order to develop new approaches, there is a need to grow a broader knowledge of vector biology which can be done by sequencing the whole genome of the mosquito to know about the vector's biological processes that can be helpful to exploit and disrupt/eradicate these mosquito borne diseases.

Sterile insect technique (SIT)

The Sterile Insect Technique, also identified as the Sterile Insect Release Method, is a method for the management of key insect pests like mosquitoes, flies. The Sterile Insect Technique is defined as "a method of pest control using area-wide releases of sterile insects to reduce reproduction in a field population of the same species". It is therefore a type of "birth control" in which wild female insects of the pest population do not reproduce when they are inseminated by released, radiation-sterilized males. In this type of autocidal control, sequential releases of the sterilized insects in adequate sterile to wild male over flooding ratio's lead to a reduction in pest population numbers. Effective control using sterile insects is achieved with area-wide integrated pest management (AW-IPM) programmes.

At present many genetic control methods are investigated for the control of mosquitoes, which include release of wolbachia infected mosquitoes and other strategies [28]. Sterile insect technique (SIT) is also another popular technique for vector control development. There are two types of approaches – conventional and transgenic. SIT involves release of sterile males which can reduce population by creating persistent infertility [29]. Conventional method includes creation of dominant lethal in gametes due to irradiation which is generally induced by gamma rays. This method generally involves somatic damage that impairs male mating competitiveness against wild males [30]. Transgenic approach involves insertion of dna encoding for I-Ppol, a homing endonuclease extracted from *physarum polycephalum*. The expression of I-Ppol eliminates holandric X chromosomes thus causing male bias progeny. Transgenic I-Ppol mosquitoes carry a visible fluorescent dye (RFP) and marked fluorescent spermatozoa which facilitates male competitiveness and survival [31]. Infertility was induced in I-Ppol male's cage populations of stable-age distribution mosquito populations. The experiments conducted in large cages particularly confirm the suitability of the beta2-Ppo2 strain for consideration in sterile insect technique programs [32]. But life history analysis of the beta2-Ppo1 and beta2-Ppo2 lines revealed retarded development of the transgenic individuals and low adult eclosion in transgenic males [32].

Transgenic mosquitoes

The genetic transformation of mosquitoes to make them refractory to *Plasmodium* infections is regarded as a potential strategy to control malaria transmission. Three key components are needed for a successful mosquito transformation [33]. First, an efficient germ line transformation system has to exist. Secondly, suitable promoters that will drive stage, tissue, and sex-specific expression of anti- *Plasmodium* genes need to be selected [34]. Thirdly successful mosquito transformation in the selection of effector genes that either impair parasite development or serve as parasitocidal agents with 100% efficiency and a low fitness cost [35].

Means of driving a refractory construct through a vector is the most problematic one [36]. Transposable elements such as the p elements are gaining attention at present as they are able to spread quickly globally by replicating within a host genome and hence inherited in off-spring's genome [37]. They are successfully implemented in *Drosophila* but the same cannot be repeated in *A. gambiae* as its activity decline substantially with increasing size and over time. MEDEA (maternal-effect dominant embryonic arrest) elements are under development which are selfish genetic elements that spread rapidly by

distorting its own off-spring's ratio. MEDEA encodes a maternally expressed toxin and zygotically expressed antidote. The toxin causes the death of the progeny [38]. Several refractory genes are to be found out to improve the efficacy of refractoriness and reduce the probability that give resistance to anti-pathogen genes will emerge in plasmodium population [38]. MEDEA elements should also be introduced into mosquito populations and their ill-ailments should also be found out by conducting large cage trials [38].

Previous research in controlling malaria was done on plasmodium parasite and anopheles mosquitoes. The drug and vaccines developed have failed to induce sufficient long lasting protective immunity. The spray of pesticides also failed because the mosquitoes develop resistance to the pesticides. So currently SIT (sterile insect technique) is gaining importance which involves release of large number of sterile individuals to cause mosquito population suppression [39]. Germ line transformation of vectors has become more common and this technology involves germ line insertion of transposons containing genes of interest in the presence of transposase enzyme [39]. The ability to rear the mosquito population of a particular species. The ability to induce sterility. Many mechanisms include chemo-sterilization, cytoplasmic incompatibility but most commonly used mechanism is the exposure to gamma radiations, which causes chromosomal breaks in the germ cells. A wide knowledge on population dynamics is also required and viability as transgenic SIT mosquitoes. Irrelevant transposon combination also causes transgene instability [39].

As we need to separate the 2 sexes prior to release, mechanical separation methods cause physical damages and reduced competitiveness constantly [40]. Alternative methods include making genetic sexing strains but it also has fitness problems, semi-sterility, and also absence of complete sterility [39]. Transgenic markers like EGFP, RFP are used to allow sex separation based on fluorescence. The sperms of male were marked with EGFP+. Mechanical-opting sorting system is used to differentiate them phenotypically. Many insect resistance genes are also used in males [41]. In case of females, many dominant lethal genes are induced, which control the transcriptional process of certain genes, instead of gene of interests are transcribed. Using transgenesis to create strains of Anopheles suitable for SIT could potentially offer several advantages over current approaches, in that the basic design of transgenic constructs designed for other insects should be rapidly transferable to mosquitoes, and induction of sterility as a product of the transgenic modification could obviate the requirement for radiation and its associated deleterious effects [39].

Transgenic strategies of controlling mosquitoes can be used in two ways. It is population suppression and population replacement. However approaches aimed at population suppression is assumed to lose transgenes after the releases are end due to environmental non-compatibility [42]. So here Skeeter buster model is being used for suppressing adult female mosquitoes. A transgene encoding a toxic protein is controlled by a promoter which turns on expression in female flight muscles. Females with dead flight muscles cannot feed, mate, fly and they gradually die [43]. Construction of this FK strain proposes a deployment method where eggs would be transported throughout a city. Local stakeholders would then be responsible for hatching eggs in suitable habitats so that FK adults would emerge but only males would be viable. These males would then mate with wild-type females, whose female progeny would not develop into viable, virus-transmitting adult females [43]. *A. aegypti* is particularly amenable to this approach because its eggs are desiccation resistant and can remain viable for several months, making them easy to transport, distribute, and hatch simply by immersing in water [44]. Here they show that population elimination might be an unrealistic objective in heterogeneous populations. They demonstrate that substantial suppression can nonetheless be achieved if releases are deployed in a uniform spatial pattern using strains combining multiple lethal elements, illustrating the importance of detailed spatial models for guiding genetic mosquito control strategies [44].

Transgene introduction in mosquitoes for population suppression or inhibition of ookinete development is also an attractive approach for the control of vector borne diseases. But the problem is that these transgenes are facing fitness problems gradually over time due to various factors [40]. So here a transgenic *A. stephensi* that express a catalytically inactive phospholipase gene (mPLA2) under the control of a midgut promoter is created. They created two transgenic lines of *Anopheles stephensi*, a natural vector of *Plasmodium falciparum*, which constitutively secrete a catalytically inactive phospholipase A2 (mPLA2) into the midgut lumen to interfere with *Plasmodium* ookinete invasion. Experiments show that both transgenic lines expressing mPLA2 significantly impair the development of rodent malaria parasites, but only one line impairs the development of human malaria parasites. In addition, when fed on malaria-infected blood, mosquitoes from both these transgenic lines are more fecund than non-transgenic mosquitoes. Consistent with these observations, cage experiments with mixed populations of transgenic and non-transgenic mosquitoes, show that the percentage of transgenic mosquitoes increases when maintained on *Plasmodium*-infected blood. Expression of an anti-*Plasmodium* effector gene gives transgenic mosquitoes a fitness advantage when fed malaria-infected blood.

Vaccines

Inactivated sporozoite immunization studies in 1942 showed a hope of combining both cellular and humoral immune responses against malaria of domestic fowl [45]. Almost at the same time, Freund was developing a powerful adjuvant that showed decent efficacy in malaria studies [46]. However, today, there is still no licenced vaccine against malaria or any other parasitic disease of humans and no deployed subunit vaccine for any parasitic disease [47, 48]. Most of the vaccines that are available today belong to

one of three categories-attenuated microbes, killed microbes or protein subunits. Attenuated viruses that protect against a cross-reactive pathogen originate with Jenner's use of a related poxvirus to prevent smallpox. After about a century, killed microbes were introduced and several such vaccines, e.g. polio vaccine, have been used widely. More recently, conjugate vaccines against encapsulated bacterial pathogens have been developed and have been hugely successful in reducing the incidence of some diseases. However, protein based licensed subunit vaccines are very few and these are particulate. Recombinant proteins that are not particulate have rarely become effective human vaccines. Parasitologists trying to develop vaccines can hardly ever safely grow and manufacture whole parasites in sufficient numbers to induce immunity, although recently this has been attempted for malaria [49]. Instead, a large number of antigens have been expressed, mainly as proteins and less often from vector systems to try to generate protective immunity. Parasite vaccines generally face the challenge of generating immunity with an immunogen that reflects only a tiny fraction (less than 1%) of the composition of the organism, a challenge that has been met only rarely in vaccinology [50]. Encouragingly, in at least one system, vaccination of pigs and cattle against *Taenia*, vaccination with a single antigen has prevented cysticercosis caused by cestode parasites [51].

Although most residents develop natural immunity to malaria in endemic areas, this usually takes a few years of exposure and is imperfect. Natural immunity predominantly targets a wide variety of blood-stage antigens and no antigen appears to be especially important in providing protection [52]. Vaccine candidates selected for vaccines are malaria antigens as they are the targets of natural immunity and significant genetic polymorphism, and a key blood-stage antigen, *P. falciparum* erythrocyte membrane protein-1 (PfEMP1), even shows temporal switching of variant expression. *Plasmodium* parasites express substantial stage-specificity of antigen so candidate vaccines for one stage of the life cycle are unlikely to impact on another stage.

RTS,S, a *P. falciparum* CS protein was showing great efficacy in sporozoite challenge studies in a novel adjuvant [53]. This RTS,S candidate has led the way for many years, showing efficacy in progressively younger subjects and in a variety of epidemiological settings culminating in an ongoing licensure trial [54]. Blood-stage vaccine candidates continue to struggle with adjuvant formulations and limited immunogenicity [49].

However, some important questions remain about the efficacy and utility of this vaccine for malaria control, not all of which will be answered by the current phase III trial. These include the level of efficacy against severe malaria, which could be higher than that against clinical malaria, the duration of protection provided by the vaccine, which was limited with the AS02 formulation both in phase IIa and phase IIb studies, and the cost-effectiveness and community acceptability of deploying a vaccine with limited efficacy [55].

Whole parasite vaccines

A major effort has been made to develop a pre-erythrocytic vaccine comprising whole sporozoites as a response to limited efficiency achieved by sub-unit vaccine candidates and RTS,S [49]. Defective schizonts can be produced by irradiated sporozoites within the liver which invade them. This can express antigens that induce a protective immune response but the main disadvantage of defective schizonts is that they cannot rupture to release the merozoites that would normally invade RBCs and continue the infection. Comparing with animal models, complete protection is readily obtainable through the activity of induced CD8 β T cells that clear infected human liver cells but this remains to be demonstrated. However, significant efficacy has yet to be reported from these studies and it remains unclear whether a needle and a syringe can substitute for a mosquito and its salivary gland fluids in generating adequate immunogenicity and efficacy in human [56]. Even if high-level efficacy can be achieved using this approach, the challenges of cost of manufacture and distribution of parasite vials in liquid nitrogen tanks in developing countries suggest that efficacy of this approach will need to be considerably higher than that of other malaria vaccines for it to be deployed widely [57].

Vectored vaccines

The other major approach to induce pre-erythrocytic immunity is to employ vectored vaccines, aiming mainly to induce cellular immunity against the liver-stage of *P. falciparum*. Irradiated sporozoite induced immunity in animal models is mainly due to CD8 β T cells and appears to target multiple antigens [57]. Using chimpanzee adenoviruses (ChAds) encoding the Thrombospondin-related adhesion protein (TRAP) pre-erythrocytic antigen to prime an immune response that is then boosted by another viral vector, modified vaccinia virus Ankara (MVA) encodes the same TRAP insert [45]. This particular prime-boost approach, first discovered in malaria, leads to much higher T cell responses than single vector immunization, and extensive studies have shown its utility in pre-clinical models [58]. A related prime-boost approach is being developed by the US Naval Medical Research Centre, but here the priming vector is plasmid DNA, and a human adenovirus, Ad5, is used to boost the immune response [59].

Blood stage vaccines

In contrast to major progress in several areas of pre-erythrocytic vaccine development, results with blood-stage vaccines have been more mixed and progress is generally slower [60]. A number of candidate

vaccines have progressed to clinical testing but they have shown limited efficacy against clinical malaria. MSP1 and AMA1 in particular are the antigens for vaccine candidates. Almost all of these candidate vaccines have been a protein given with an adjuvant designed to induce protective antibodies. Some approaches have focused on inducing antibodies that impair parasite growth, as can be demonstrated in the in vitro assays [5], whereas others have aimed to induce antibodies which achieve their effect in collaboration with effector cells and which can be measured with greater difficulty, in a functional assay of ADCl of parasite growth [61]. There have been three particular challenges for the development of blood-stage vaccines. One is difficulty in expressing conformationally correct large antigens and scaling up the methods needed to do this to the extent that would allow large-scale manufacture. A second challenge has been the only modest antibody responses achieved even with a range of new adjuvants. The third difficulty has been posed by the extensive polymorphism of many leading candidate blood-stage antigens [45].

Mosquito stage vaccines

The principle of this vaccine is that immunization with gametocyte or ookinete antigens could reduce or ablate oocyst development in the mosquitoes [62]. Membrane feeding assays are getting standardized that allow sera from vaccinated animals or humans to be evaluated for their ability to reduce or prevent transmission. The in vivo use of transgenic parasites has supplemented to assess the efficacy of antibodies induced by *P. falciparum* and *P. vivax* antigens in preventing transmission of rodent parasites [63, 64]. These systems provide means of rapidly assessing the likely efficacy of sera from vaccines participating in phase I trials of mosquito-stage vaccines, a major advantage of this approach to malaria vaccine development. In view of the potential of this approach to the prevention of malaria, it is surprising that it has been so little supported. The main hurdle has been the concern that deployment of such a transmission- blocking vaccine would prove impractical. This type of mass vaccination has been undertaken on occasion for other diseases but may prove logistically challenging.

Table 1: Comparison of different approaches to control mosquito borne diseases on the basis of effectiveness [65-69]

Method	Principal target	Approach type	Affordability	Effectiveness
Environmental control	Mosquitoes	Non-genetic	Cheap	Substantially high but slow
Biological control (Fungi)	Mosquitoes	Non-genetic	Cheap	Slow and less effective
Biological control (Fish)	Mosquitoes	Non-genetic	Cheap	Less effective
Essential oils	Mosquitoes	Non-genetic	Cheap	Considerably effective for a short span
Chemical control	Mosquitoes	Non-genetic	Considerably high	Highly effective with many disadvantages
Transgenic mosquitoes and SIT	Parasites	Genetic	High	Research in progress
Vaccines	Parasites	Genetic	High	Research in progress

CONCLUSION

Although several approaches towards controlling these deadly mosquito borne diseases are being investigated in recent times, but it is indeed necessary to look for other factors such as affordability and availability for the common person. So a combination of all these approaches can control these mosquito borne diseases unless these vectors gain resistance to them. A lot of research work is to be done in developing anti-parasite vaccines and by reducing the harmful-vector populations effectively using transgenic techniques which are major prospects of the post genomic era. As the eradication of harmful vector populations using transgenic technologies might take a very long time in order to protect ourselves we need to use many traditional non-genetic approaches like draining of stagnant water, use of mosquitocidal nets and use of essential oil mixtures for our well-being. The main important step to be initialized now is to develop a perfect model for combating these vector population which comprises eradicating future vector population using transgenic technologies and protecting ourselves by developing anti parasite vaccines and other non-genetic approaches.

CONFLICT OF INTEREST

There is no conflict of interest.

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FINANCIAL DISCLOSURE

None.

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ARTICLE

ZEBRAFISH: A VERSATILE BEHAVIORAL STUDY MODEL

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ABSTRACT

The popularity of zebrafish as a model organism can be accounted for by a number of attributes, including the relative ease of rearing and breeding in captivity, rapid development, short generation time, and availability of genomic resources, including the complete zebrafish genome sequence. Zebrafish is described as a versatile behavioral model since it is known to adapt to a wide range of changes in its surroundings without much difficulty and provides us with a reliable means of reproducing different aspects of human behavior and studying them. Though it is not only used for neurobehavioral studies, but also for several other purposes in an equally efficient manner. In this paper, we focused on recent developments made in behavioral studies using zebra fish. We tried to bring home the idea that zebra fish can be subjected to behavioral studies that may allow us to peek into the basic mechanism of some of the commonly seen behaviors of vertebrates like anxiety, social behavior, learning etc. along with the current research trends in this aspect.

INTRODUCTION

KEY WORDS
Zebrafish, behavioral study, neurotransmitter, age.

An animal model with biological and/or clinical relevance in the behavioral neurosciences is a living organism used to study brain-behavior relations under controlled conditions, with the final goal to gain insight into, and to enable predictions about these relations in humans and/or a species other than the one studied, or in the same species under different conditions from those under which the study was performed [1]. Of the many species used in biomedical research, specific animals are preferred in certain areas. Non-human primates are used in research on vaccines, infectious, cardiovascular, and neurological diseases, aging, reproductive biology, gene therapy, drug addiction, xenotransplantation (cross-species transplants), and vaccine and toxicity testing [2]. Zebrafish are vertebrates. Like humans, they have a backbone. This means that they are more closely related to humans than commonly used invertebrate models such as insects and worms (*Drosophila* - fruit flies and *Caenorhabditis elegans* - nematodes) which do not have backbones. Because zebrafish are more closely related to humans, they are more likely to be similar to them in many biological traits than a more distantly related organism. These biological traits would include genes, developmental processes, anatomy, physiology, and behaviors. This is an advantage that invertebrate lab animals do not share with humans. The invertebrates are more appropriately used in comparisons at the cellular or biochemical level of organization where they share many features with humans. The embryos develop quickly. They go from a single cell to something that is recognizable as a tiny fish within 24 hours which mice take 21 days. Although zebrafish and humans are obviously very different, their embryonic development is remarkably similar. Furthermore, it is becoming clearer that all vertebrates follow an evolutionarily-conserved developmental program. This conservation extends even to the molecular level where similar genes perform similar functions in many different species [3]. Zebrafish is a tropical fresh water fish native to the Himalayan region. Its lifespan in captivity is around two to three years, although in ideal conditions, this may be extended to five years. As a model biological system, the zebrafish possesses numerous advantages for scientists. Its embryonic development is very rapid, and its embryos are relatively large, robust, and transparent, and able to develop outside their mother. Furthermore, well-characterized mutant strains are readily available. Zebrafish is a small (3-4 cm long) freshwater teleost species that can be easily kept and bred in the laboratory [4]. A female can produce 200 eggs per spawning and the fry grow quickly and reach sexual maturity within 2-3 months. Zebrafish has been successfully used in developmental genetics and recently neurobiologists have also started to study this species [5]. Zebrafish are highly prolific, resilient, and one of the lower order vertebrate species in which complex brain function and behavior may be studied in the laboratory [6]. About, 70% of protein-coding human genes are related to zebrafish genes, and 84% of the genes known to be associated with human disease have a counterpart in the zebrafish genome. The behavioral repertoire of zebra fish is complex and should allow the development of a range of behavioral paradigms. Thus, an effort has been made in this article to highlight some of the key futuristic studies involving zebrafish to enunciate its potential as a remarkable study model for complex human behavior.

ZEBRAFISH: A PROFICIENT STUDY MODEL

The Zebrafish and behavioral studies

In this paper, we discuss some of the attempts made by several scientists all over the world to understand the neurological conditions of zebra fish by relating them to several behavioral parameters measured using a wide variety of methodologies; different stimuli bringing out different responses like anxiety, stress etc., also the effect of neurochemicals, age-related studies and their behavior in a social environment.

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Anxiety

The ultimate goal of most anxiety-related studies has been to develop zebra fish models of pathological processes and to investigate the mechanisms of fear and to eventually translate the findings to the human clinic. Different approaches have been made to study fear, stress and anxiety-related responses in zebra fish. Here, we will be seeing the different methodologies and parameters used to measure the levels of anxiety and other interesting outcomes of the experiments done till date in this aspect [7].

Three dimensional robotic models have been used, for example, to measure the anxiety-fear responses in zebra fish, live predatorial stimulus was created by designing a robot mimicking the morphology and locomotion pattern of a natural predator of zebra fish-Indian leaf fish *Nandus nandus*.

Another stimulation was in the form of an attack by a heron (a bird silhouette), impacting the water surface. In the presence of predatorial stimuli, the fish give the avoidance response. Also, the zebra fish responded to the predator fish irrespective of the color and pattern of the predator, but more to its tail frequency. Anxiety-related studies were also conducted using alarm substances, which is known to induce fear responses in a range of fish species. Alarm substance is formed in specialized epidermal club cells and released when there is a skin injury. This is detected by the neighboring fish using their chemoreceptors (olfaction), followed by decrease in the distance between fish in the shoal (a group of fish) and then moving away from the predator. Two motor patterns are known to change in response to alarm substance or other fear-inducing stimuli, erratic movement and freezing. Freezing was defined as complete immobility whereby only the gills and occasionally the eyes move. In nature, when erratic movement is performed at the bottom of small creeks or lakes, it stirs up the debris and the cloud formed covers the fish. The zebra fish show an increase in erratic movement. It is likely that in nature zebrafish exhibit the strongest alarm reaction when an injured fish is nearby and thus the concentration of alarm substance in the water detected by the neighboring fish is high. If the injury occurred farther away, the predator is unlikely to be nearby, and the smaller concentration of the alarm substance elicits a diminished alarm reaction. The presence or absence of the predator did not have any effect on the action of the alarm substance. Increased shoal cohesion is also thought to be an anti-predatory response, since fish in a shoal represent a more difficult target because the predator's attention is divided [8]. In another study [9] acute alarm pheromone exposure resulted in a significantly longer latency to explore the upper portion of the tank, frequency of freezing and erratic movements also increased. Around 7 ml of alarm pheromone solution extracted from euthanized zebrafish was added acutely to a novel test tank, 22 fish were subjected to this acute treatment. For prolonged exposure the fish were exposed to alarm pheromone in the pre-treatment beaker for 30min after which they were transferred to novel test tank, 20 fish were used for prolonged exposure. Prolonged alarm pheromone exposure did not result in any significant behavioral differences between the control and experimental groups. The results confirm that alarm pheromone each evoke relatively simple, yet robust anxiety-like behavioral responses in zebrafish. Interestingly, prolonged alarm pheromone-exposed zebrafish showed no significant behavioral differences compared to control fish. Thus, alarm pheromone appears to only be effective acutely, reflecting its natural use as a danger signal to nearby shoals. Chlorpyrifos is a common organophosphate pesticide(OP) used in the U.S. and some OPs have been found in detectable levels in air, dust and food samples and even in children's urine samples. To study the neurobehavioral disorders caused due to this exposure, zebrafish larvae are used to examine the effects of sub-chronic levels of chlorpyrifos on anxiety-related behavior during their development. Zebrafish larvae develop rapidly, hatching from their chorions by 2–3 days post fertilization (dpf), and fully develop all adult organs within their first week. By 4–5 dpf, larvae inflate their swim bladder and become free-swimming, exhibiting a number of behaviors such as avoidance, darting, scoots, and startle response. Thigmotaxis, a preference for the edge, is an anxiety-related behavior in zebrafish larvae. The highest dose of chlorpyrifos administered, 1 μ M, had a highly toxic effect on movement, body morphology, and body size. At this dose, the larvae exhibited twitching behavior but could not swim or move normally; therefore, they were not used for behavioral analysis. At this concentration, zebrafish larvae exhibited tails that curled upward and shorter than normal body lengths [10].

In one of the experiments, images were used as stimuli since olfactory cues (like alarm substance) are difficult to turn on and off as and when required and using a live predator may create an error variation (like if it actually attacks the prey fish). The images included the original sympatric predator, the Indian leaf fish, another sympatric predator, the needle fish, a bird silhouette moved on the side or above the tank, an expanding dot mimicking rapid approach of an object shown on the side and from above the tank, as well as non-fear inducing images including a single and a group of zebrafish. Another type of behavior observed in response to fear-inducing stimuli was jumping, a single fast jump with the use of the caudal fin. Similarly, 'leaping' also occurred (the attempt to perform a forceful and fast swim-like jumping) against the glass. Freezing is usually different from floating, which can occur anywhere in the tank but freezing occurs only at the bottom of the tank or when the fish is in contact with an object. Another form of active swimming is thrashing. It is a forceful swim that appears as if the subject was trying to swim through the glass of the tank and manifests as circular motion directed towards the glass. The fear response to needle fish was not that effective except for a few jumps and leaps. Even the image of Indian leaf fish or the dot on the side were not as effective as the dot approaching from above in eliciting fear responses. Fish respond to approaching predators with fleeing, fin erection display, or can even ignore the predator depending on the distance between them and the predator, or on the level of satiation of the predator and many other factors including access to escape routes. The reduction in mobility could also be due to non-

threatening stimuli and time spent by the fish at the bottom of the tank could depend upon external factors [11]. When given free choice between a black and a white chamber, zebrafish reliably demonstrate a preference for the black chamber, and in analogy to rodent models, it has been suggested that the degree of preference may be useful as a measure of anxiety. According to a hypothesis zebrafish may have its light/dark preference varied with the circadian clock [12]. The hypothesis was tested by recording the light/dark preference in a tank was divided into two compartments, one was illuminated by LED light and the other was covered with matte black paper on all sides and the top. The LED light was at the top of the experiment tank. A video camera was positioned in front of the tank. The behavior of zebrafish was recorded from 8:00am on the first day to 20:00pm on the third day. Between 9:00 and 20:00 during the third daytime melatonin was added so that the final concentration of melatonin in the tank was 0.1 mM. On day 1, the fish displayed a preference for the light area of the aquarium between 8:00am and 18:00pm. This preference was reversed to dark tendency when the time was 20:00pm. The preference for dark region continued till 2.00 am next day and thereafter started to decline reaching the minimum value at 8.00 am. The same preference continued for the next 24hr. 0.1mM melatonin significantly increased the mean proportion of time in the dark to over 70% at 10:00am. These results indicate a clear circadian-like tendency of staying in the dark area also melatonin treatment indicate that Melatonin could mediate the circadian clock of zebrafish. Behavior of zebrafish influenced by melatonin is dose dependent. Zebrafish adults displayed higher activities during the daytime, as they need to detect predators, to get food or to find conspecifics. In the nighttime, fish displayed brief periods of inactivity, often associated with a drooping caudal fin, suggesting a sleep-like state. During the night time, they prefer dark backgrounds to minimize their visibility and their locomotor activities. This study can aid in providing new insights in understanding the metabolism mechanism underlying the neurobehavior, and facilitate studies related to the neurobiology of normal and pathological behavior. There is still doubt regarding whether the zebrafish find the white compartment aversive or it is that they like dark compartment more. To check this a study [13] was conducted wherein avoidance of the white compartment was studied by analysis of the time spent in the white compartment of the apparatus and shuttle frequency between compartments and thigmotaxis, freezing and burst swimming in the white compartment. This study consisted of 3 experiments, in the first experiment a preference tank was divided into 2 compartments black and white. Fish were first kept in a central compartment for 5min habituation period then were allowed to make an initial choice between the two compartments. The behavior was video recorded for 15min. 3 such trials were conducted. During the inter-trial time of 15min the fish were kept in the acclimation tank from where they were taken. Experiment 2 was same as experiment 1 only difference was that the inter-trial time was 24hr. 5 trials were done. In experiment 3- 10 fish were individually transferred to the white compartment and were not allowed to leave the compartment for 15 min. After this treatment they were again subjected to experiment 1. The results indicate that forced exposures (post-confinement trials) did not alter the time spent in the white compartment or general locomotor activity (shuttle frequency), but it decreased the proportion of time that animals spent burst swimming, sticking to the walls, or freezing while in the white compartment. The results could indicate that exploration of the white compartment is driven primarily by fear/anxiety than by novelty.

Preference for dark place may be a measure of anxiety but which measures can be considered as a measure of anxiety is not clearly understood, in a study [14] Subjects were observed individually in a single session lasting approximately 2 h. The session included a preliminary black/white preference test, forced exposure to black and to white, and a final black/white preference test. Each one of the test lasted for 30 minutes. Subjects spent on average 34% of the pre-test in white and on average 36% of the post-test in white compartment. 21 of the 62 subjects spent 50% or more of the preference tests in the white compartment, while 41 spent less than 50%. Most of the fish preferred the black chamber to the white chamber as predicted which replicates the results of previous experiments. Freezing is the primary response of zebrafish to stimuli that are avoided this is evident from high levels of freezing observed in fish which avoided white chamber in pre-test and were subjected to it in the second test. So freezing can be proposed to be the most reliable behavioral measure of anxiety.

Zebrafish is a shoaling fish and a study [15] was conducted to check how shoaling is related to anxiety and stress The study focused on the effects of a variable number of *Danio rerio* fish subjects, ranging from 1 to 8, in the light/dark box preference test. There were four groups in this experiment and a different number of subjects was used in each group: the control group had only one subject inside the apparatus during the test, whereas the experimental groups had 2, 4 and 8 subjects. The subject's behavior is recorded with a video camera during 15 min after a 5min habituation period. There was reduced white avoidance behavior in the group of eight subjects reflects the role of shoaling which is a defense mechanism in reducing anxiety and stress. However the test results show that four subjects inside the apparatus is not enough to cause the anxiolytic effects of shoaling in the light/dark test. The absence of significant difference between the control group and the test groups with two and four subjects suggest feasibility to run the light/dark test with up to four subjects.

Anxiolytic effects

In novel environments, zebrafish swim to tank bottoms and dark backgrounds, which is attributed to anxiety associated with the threat of predation. AB, WIK, PETCO and Globish strains of zebrafish were treated with different drugs like nicotine, desipramine, chlordiazepoxide, yohimbine, citalopram, DMSO and ethanol and subjected to two novel environments: dive tank top and light-dark plus maze. The dive tank anxiety list is based on the tendency of zebrafish to remain in side and bottom contact with solid

boundaries in a novel tank and the aquatic light/dark plus maze is based on the tendency of zebrafish to seek dark backgrounds (or avoid light backgrounds) in unfamiliar environments. In the novel dive tank test, WIK line spent significantly more time in the top 2/3 of the dive tank than the others. This might be due to absence of predation pressure in their natural Indian Wild population environment. A moderate dose of nicotine increased the amount of time the fish spent at the top of the tank while higher amounts decreased mobility, indicating the possibility of sedation at higher doses. Chlordiazepoxide failed to increase zebrafish dwelling at the top but increased the amount of time on the light side and the serotonin reuptake inhibitor citalopram increased the amount of time spent by the fish in the upper sections of the tank. Exposure to ethanol or anxiolytic drugs reduces the light avoidance behavior. Zebrafish can be used for drugs or toxin screening. Update of water-soluble drugs into zebrafish brain through bath exposure produces brain drug concentrations that are roughly 1/1000 of bath concentrations [16]. To understand the behavioral and physiological stress in zebra fish, tank dive test and treatment with pharmacological substances were performed. Alarm pheromones and caffeine used were anxiogenic (causing anxiety) and fluoxetine and ethanol were anxiolytic (reducing anxiety). The extent to which acute and chronic exposure to caffeine and fluoxetine caused behavioral modulations was also observed. Alarm pheromone increased the frequency of erratic movements and freezing behavior. Caffeine increased the latency (hesitation) to enter the upper half, also increased erratic movements. Chronic exposure to fluoxetine caused lower latency to enter the upper half and treatment with ethanol increased the amount of time spent in the top. Also, zebrafish physiological stress responses were measured using a cortisol assay. Chronic exposure to fluoxetine showed reduced levels of cortisol, which indicates that it reduces anxiety [17].

Alcohol studies

Zebrafish is a potential model for analysis of drug addiction, e.g. alcoholism. Ethanol can easily cross biological membranes and affects virtually all body organs. Behaviorally, acute administration of alcohol in humans leads to disinhibition and euphoria. The use of animal models with similar or related behaviors may provide insights into molecules involved in mediating the biological effects of ethanol. Zebrafish are more structurally homologous to humans, and their genes are about 70–80% identical to human counterparts thus making it convenient to identify human orthologues of zebrafish genes. Although zebrafish have been widely used for studies of development, their use in the study of the effects of ethanol has been limited.

Study has been reported on basic behavior tests that account for characteristic behavioral patterns of zebra fish upon alcohol treatment where fish were tested in 6 behavioral paradigms-locomotor activity, group preference, aggression, anti-predatory model, light/dark preference, pigment response [4]. About 4 concentrations of ethanol were employed - 0.00%, 0.25%, 0.50%, and 1.00%. For locomotor activity fish treated with 0.25% and 0.50% showed significant increase in activity as compared to fish treated with 1.00% or control fish also fish treated with 0.50% alcohol spent more time in the upper layer of water than other fish. Fish treated with 0.00% or 1.00% alcohol spent significant amount of time in lower layer of the tank. Zebra fish generally swims near the surface of water. It only goes to the bottom when there is a threat from predators like birds. Control fish swam near the bottom indicating anti-predatory behavior but fish administered to 0.50% were generally in the upper layers initially this indicates that alcohol has anxiolytic i.e. anxiety reducing effect. For group preference alcohol significantly reduced the preference for conspecific in a dose dependent manner, for anti-predatory model 0.25% showed the strongest jump response. For light/dark preference test during the 1st minute of observation all fish avoided the dark component. But after 10 minutes of habituation 0.00% and 0.25% fish didn't show any preference or avoidance for any compartment but 0.50% and 1.00% showed avoidance for dark compartment [4]. This suggests that alcohol likely to affect central neural mechanisms rather than perception. For pigment response Alcohol enhanced the color of zebra fish, there was a linear relationship between alcohol concentration and skin color. It was speculated that alcohol may directly act on chromophore cells and change color or indirectly act by affecting the central neural mechanisms [4]. So it can be concluded that lower doses of alcohol had facilitatory role and higher doses had inhibitory dose and Alcohol likely to affect central neural mechanisms rather than perception.

Another study has been reported [5] where the aim was develop a method for inducing voluntary ethanol intake in individual zebra fishes which can be used as a model in future studies, and to characterize the effects of ethanol intake on different behaviors and the expression of hypothalamic orexigenic peptides, galanin (GAL) and orexin (OX) [5]. Gelatin-ethanol meals were prepared which consists of melted gelatin mixed with 2 day old brine shrimp plus ethanol (in three different concentrations) which resulted in three different types of meals with ethanol concentration being 0%, 10%, 20%. Gelatin-ethanol meals were prepared fresh daily. The increased intake of ethanol-gelatin may not due to an ethanol-induced change in appetite. Instead, zebrafish may have a preference for the pharmacological effects or the taste of ethanol-gelatin, at least at the 10% concentration, which in humans and rodents is perceived as containing both sweet and bitter taste components. The ethanol-gelatin caused a significant increase in locomotion and a decrease in anxiety, as indicated by increased exploration in zebrafish that ingested the 20% ethanol-gelatin. Voluntary intake of ethanol-gelatin stimulates the expression of the orexigenic peptides, GAL and OX, in the hypothalamus of zebrafish. This effect was found to be anatomically specific, occurring for GAL in the ventral and caudal zones of the periventricular hypothalamus, with a significantly greater effect in the caudal compared to ventral zone, and for OX (+60%) in the anterior periventricular hypothalamus.

Another study focused on acute effects of alcohol on larval zebrafish: a genetic system for large-scale screening [18]. The effects of acute treatment of alcohol on locomotion, thigmotaxis (wall seeking tendency in an open field) and melanocyte morphology in larval zebrafish were studied. Two strains of zebrafish were used AB (originated from Oregon, USA,) and WIK (originated from Germany). Larval zebrafish were obtained from these strains through natural mating. 7-day old fry (larval zebrafish) were used in this study. Larval zebrafish exhibits acute sensitivity to ethanol in a dose- and time-dependent manner. They initially become hyperactive, and as ethanol accumulates, they become hypoactive and sedated. This is similar to what has been observed in humans and other animal models. Also since the activating time of ethanol took several minutes the effect of ethanol on behavior is likely to be mediated by CNS than by chemosensory pathway because chemosensory pathway takes very less time. Future forward genetic analysis to identify mutations that show altered sensitivity to ethanol shall provide important insights into the genes involved in regulating the behavior. Another experiment with larval zebrafish included studying the swimming pattern of 5- dpf (days post fertilization) zebrafish larvae which were exposed to light in a particular sequence which was bright dim-bright. This experiment was to check a hypothesis that untreated fish will show more activity at the bright-dim transition as compared to dim-bright transition whereas ethanol treated fish will show more activity on both light transitions. 27 AB strain (9-10dpf) larvae were taken of which 15 were control i.e. they were not subjected to ethanol treatment rest 12 were exposed to 12% ethanol for 30 min prior to experimentation. Fish were first acclimated to bright light for 5 minutes and then abruptly switched to dim light for 15 minutes, followed by a transition to bright light for 5 minutes. Fish were placed in a standard 24-well plate (one fish per well). For bright-dim transition 9 out of 15 control fish responded within 5000 milliseconds of the change and 11 out of 12 ethanol treated larvae responded within the same time frame. For Dim-bright transition - Only six out of 15 responded however all but one ethanol treated fish responded with latency less than 5 seconds. The results confirm the hypothesis, also the ethanol treated fish were quicker to respond to light transition as compared to control fish. The larval zebrafish is an excellent model for investigating locomotory kinetics and drugs with anxiolytic properties and change in melanocyte morphology.

Learning

There is lack of research on alcohol dose-effect and how it acts on the brain. The drug seeking behavior caused by different alcohol doses in short and long term uses and the effects of different alcohol doses on a learning task [Fig. 4] with a cognitive element was tested in an study were lower doses (0.10%) resulted in learning behavior and no seeking behavior was generated [19]. Higher doses (0.25% and 1.00%) impaired the associative performance and induced search for the drug. low alcohol doses (0.10%) were able to learn to associate stimuli at least 2 days in advance of the control group. On the other hand, 0.25% and 1.00% chronic alcohol treatment inhibited learning behavior. Only 0.25% and 1.00%, both in acute or chronic use, generate alcohol seeking behavior. The 0.25% group showed worse learning performance than control and 0.10% groups. This study confirms the importance of zebrafish as a model for drug throughput screening. Zebrafish can be used for designing models to reverse drug seeking behavior such as punishment or reinforcement associated to withdrawal in order to weaken the brain reward systems. Also studies which show changes in brain because of low and high alcohol doses must be done.

In another study associative learning in zebrafish in the plus maze was studied [20]. Two classical learning tasks in a plus maze were employed. In the first task zebrafish were required to associate a visible cue with food reward irrespective of the location of this pairing. The visual cue was a red plastic cue card as it is expected to be clearly distinguishable for the tetra chromatic zebrafish. In the second task, zebrafish were required to find the fixed location of the food reward. The location of this reward was not marked by an intra-maze visible cue but instead it was supposed to be identified based upon external visual cues that surrounded the maze. Both groups showed some preference toward the target arm. This may be because the target arm was the only location where food was accessible and thus zebrafish may have spent slightly more time there actually eating the food. The zebrafish that received the visual cue-food reward pairing responded with strong preference towards the visual cue alone during the probe trial, and the fish in the unpaired group did not. Zebrafish appeared active, explored the plus maze, and exhibited no signs of fear. Zebrafish is capable of attaining good performance in these associative learning tasks. The significant increase of time in the target arm demonstrated that zebrafish of the paired groups have learned and remembered the association between the single visual cue and the food reward (simple associative learning), and in the following task, the location of the food reward (spatial learning).

Neurotransmitter studies

Dizocilpine also known as MK-801, is an uncompetitive antagonist of the N-Methyl-D-aspartate (NMDA) receptor, a glutamate receptor discovered by a team at Merck in 1982. Glutamate is the brain's primary excitatory neurotransmitter, it has been utilized in the analysis of mammalian learning and memory. The zebrafish is novel vertebrate study species that has been proposed for the analysis of the mechanisms of learning and memory.

In a study, the suitable dose of MK-801 that does not elicit performance impairing effects, a concentration that may be appropriate for the analysis of learning and memory in zebrafish [21]. It was investigated whether MK-801 can disrupt motor function (important for navigating through the maze), visual perception (whether the drug can disrupt the ability to see the target stimulus), and motivation (whether the drug can

reduce/alter shoaling tendencies) in zebrafish. Four concentrations of MK-801 hydrogen maleate was dissolved in system water: 0 μ M, 2 μ M, 20 μ M and 100 μ M. The drug was administered at three different time points: (a) fish received the drug during the 30 min long behavioral session, (b) 30 min period immediately before the behavioral session, and (c) 30 min 24 h before the behavioral session. 3 behavioral tests were conducted: open tank, light-dark preference, and group preference. All tests were conducted between 10:00am and 4:00pm. The behavior of the fish were video recorded. The recording session was for 30min. MK-801 in the currently employed dose range (0–100 μ M) appeared fairly safe, led to no increased mortality or morbidity, and rarely resulted in significant behavioral changes. Most of the motor and posture patterns of zebrafish remained unaltered by MK-801 in the open tank. Results suggest that even in the larger rectangular tank, circling may be induced by MK-801. The behavioral and neurobiological mechanisms underlying this drug response are unknown at this point. Lower doses of MK-801 are unlikely to alter vision and the motivation to swim close to conspecifics. Results suggest that the highest safe dose that is unlikely to affect motor function, visual perception, and/or motivation to respond to conspecifics of zebrafish is 20 μ M, and MK-801 at this dose will not affect these crucial performance factors irrespective of whether the drug is present during or if it has been administered prior to the behavioral test. Therefore it is suggested that 20 μ M may be an appropriate concentration for the analysis of the potential learning and memory impairing properties of MK-801 in zebrafish. In another study [22] effects of acute exposure of dizocilpine [(+) MK-801] on behavior was studied. 3 experiments were conducted. Experiment 1- In this experiment- 3 round chambers were taken of which one contained deionized (control) water, other two containers had 2 μ M and 20 μ M (+) MK-801 hydrogen maleate respectively. In each tank 10 fish were kept. After 5min habituation period observations were made. Experiment 2- Before experiment male and female fish were subjected to 0, 2, 20, 200 μ M of (+) MK-801 for 1hr. Subjects were then placed in observation chambers and activity was measured. Swimming activity was monitored by counting the number of line crossings in a 30-s observation period every 6 min. Experiment 3- In this experiment fish was placed at the start point of the T-maze and allowed to freely explore the maze for 5 min. The time of entry into the EC (Enriched chamber) was noted only after a fish spent 20 consecutive seconds in the chamber. Additionally, total time spent in the EC was recorded to determine chamber preference. Readings were taken at 27 (Trial 2) and 48 (Trial 3) h post dosing. Measurements of circling, swimming activity, and preference indicate (+) MK-801 exposure associated modification of behavior in zebrafish. Treated fish circled almost continuously, while control fish were active but rarely completed a 360° rotation of the test chamber. There was a trend for control fish to reduce the time that it took for them to reach the EC during the second and third trials. In contrast (+) MK-801-treated fish did not express a preference for the EC, nor were there significant differences in time to reach the chamber across trials. A preference for the chamber may provide the necessary motivation for route acquisition. The results of these experiments suggest the utility of further investigation of zebrafish as a potential model organism for assessing both normal and dysregulated glutamate systems.

Another category of behavioral studies includes analyzing the effect of neurochemicals on several characteristics of zebra fish. Since all of the embryonic development occurs outside the mother, zebrafish are being widely used to study neurodevelopmental defects associated with toxicant exposure and neurological diseases [23].

In one of such experiments, the effects of neurotransmitters like DOPAC and 5-HIAA on the serotonergic and dopaminergic systems of zebra fish and how it reflects on the maturation of shoaling in zebra fish. This development is compared among two different strains of zebra fish. Shoaling behavior is defined by the amount of Inter-Individual Distance (distance between each focal fish), which is expressed relative to body length. HPLC was performed to find out the amount of dopamine and serotonin and their respective metabolites DOPAC and 5-HIAA since previous studies indicated that dopaminergic system is involved in shoaling in zebrafish and serotonergic system is involved in fear- which induces shoaling. The differences in shoaling behavior of the strains AB and TU were studied on different days post-fertilization. These differences might be due to differential development or growth rates. But when the body length was measured, identical growth was recorded in both strains. From 7 dpf to 87 dpf, zebrafish reduced their inter-individual distance within the studied ten member shoals from about 14 body lengths to about 6-7 body lengths. The rapid increase of shoaling in TU coincided with the step-wise increase of dopamine and DOPAC levels seen after 40 dpf. The steady increase of shoaling seen in AB coincides well with the linear age-dependent increase of dopamine and DOPAC obtained from this strain [24]. In another study [25] animated images of conspecific are shown to fish conspecific means member of the same species, presentation of conspecific stimulus fish has been shown to be rewarding in zebrafish. Dopamine plays important roles in motor function and reward. Zebrafish have dopamine receptors homologous to mammalian counterparts, and dopamine receptor antagonists. Animated images act as a visual stimulus and the effects of it are monitored and the amount of dopamine, DOPAC, serotonin and 5HIAA extracted from the subject's brain immediately after the stimulus presentation using HPLC with electrochemical detection are quantified. Instead of animated images live conspecifics can also be used but animated images have an advantage that they are consistent and experimentally well controlled. Experimental subjects presented with zebrafish images all significantly decreased their distance to the computer screen as compared to the no stimulus group. Fish presented with zebrafish images for 10 or 15 min had significantly higher dopamine levels as compared to the other three groups. Fish that received the scrambled image had significantly lower levels of 5HIAA than all other fish, while other groups did not differ from each other. DOPAC levels significantly increase in response to the presentation of zebrafish images but not in response to the scrambled images. Serotonin levels significantly decrease in response to the presentation of the scrambled without the zebrafish images fish had no preference towards any side of the

tank. However upon presentation of conspecific images the distance from the screen decreased. Elevated dopamine and DOPAC levels induced by the sight of conspecific images are the result of increased dopaminergic function, which is due both to elevated dopamine release (synaptic transmission) and to increased dopamine synthesis. It is the appearance and not the mere presence of the social stimulus that engages the dopaminergic system. Future study in this field should focus on questions like How other neurotransmitter systems may be involved in shoaling in zebrafish and also importantly what neural circuits, and in general which brain areas, may be involved? In another experiment, different types of neurochemicals were tested on zebra fish. Chlorpyrifos exposure [23] has been shown to impact dopaminergic, serotonergic and noradrenergic systems. Early developmental exposure to a specific dose of CPF has been shown to cause a learning impairment. Nicotine and pilocarpine exposures were used to determine the contribution of the receptor systems to the CPF-induced effects. Startle response and habituation over a period of 10 minute trials were used as a measure of the effects of these chemicals. The startle response was induced by a tap. Developmental chlorpyrifos exposure caused an overall increase in startle response in adult zebrafish but only different higher doses of nicotine and pilocarpine increased startle response, though the response had more significance than the DMSO controls, showing that CPF exposure causes the fish to not get habituated to the tap-stimulus.

Social behavior related studies

Social behavioral study using zebrafish can be done to gain more insight regarding autism and schizophrenia where social behavior impairments are prominent [26].

Zebrafish (*Danio rerio*) are visually drawn to conspecifics (related to the same species) [26] and adults instinctively aggregate into shoals. They are highly social animals that live in groups with structured social relationships including shoaling, dominance hierarchies and territoriality [27]. Shoaling is one of the most important social behavior exhibited by zebra fish. It is thought to provide the individual fish with multiple benefits including access to mates, efficient foraging and defense against predators [28]. Shoaling is usually measured in terms of body lengths. The performance among zebrafish strains: inbred (AB) or wild-crossbred (WIK) from Zebrafish International Resource Center, to golden and short-fin from Petco stores, was compared to study the social interaction and novelty preferences among zebrafish [26]. AB is the oldest inbred strain, WIK is recently obtained from wild-caught zebrafish, short-fin zebrafish are outbred and golden form occurs due to a pigment mutations in short-fin.

Social interaction test: In this study, colorless water-filled acrylic box with stranger fish and blue water-filled acrylic box without a fish were placed at opposite ends. AB fish spent more time with the stranger fish and Petco short-fin zebrafish spent more time in the empty blue box than the other strains. This behavior of Petco short-fin zebra fish could be attributed to their environment during juvenile imprinting [26].

Social novelty preference test: Instead of blue box, a colorless box containing a 'new' stranger zebrafish was used while the old stranger was placed at the opposite end. AB and golden mutant strain preferred the new stranger while Petco short-fin fish spent an equivalent amount of time with both the old and new stranger zebrafish [26].

Social preferences: Shoaling preferences emerge during juvenile phase and are visually mediated, so that when given a choice between shoal mates with different coloration patterns, individuals prefer to shoal with those sharing the same coloration pattern as the fish with whom they were raised. Once established shoaling preference remains stable and it is not reversed by changing their social environment.

Social recognition: Zebrafish use both visual and olfactory cues in social recognition. Studies showed that visually mediated recognition is based on a mechanism of phenotype matching against a learned template in early life. Olfaction also plays a role in species recognition as well as kin recognition in zebrafish, through a process of phenotype matching.

Social learning: Groups of zebrafish learn an avoidance response to an electric shock faster than single individuals. It has also been shown that zebrafish can learn escape routes from trained demonstrators, and that the presence of demonstrators in groups of naïve individuals increased the escape response [27].

There also have been other experiments which demonstrate social learning among zebra fish. One of them was to find out whether zebrafish at the center of their social networks have a greater impact on group behavior than less socially connected individuals. 'KEY' individuals are the most central to the groups because they interact readily with most of the other individuals, whereas 'NON-KEY' fish either stay at least two body lengths away or remain in the proximity of one other fish. Both the key and non-key fish were recognized and taken into another tank with an opaque screen leaving a gap enough for them to pass through to the other side. When the key and non-key zebrafish were placed in different groups in similar tanks, a wooden stick was introduced into the tank to induce avoidance behavior. This was done in three trials. The group with the key fish was faster to cross to the other side than the other groups with the Non-key fish in all the trials. This supports the idea that group motion is, in part, an indirect consequence of social relationships [29].

Age related behavioral studies

These studies consist of observing different behavioral parameters that change with age. For example, locomotor behaviors of zebra fish larvae have been observed. These studies focused on activity and space use in zebrafish larvae from 4 dpf to 7 dpf. Two experiments were conducted. In Experiment 1, the same larvae were observed from post-fertilization day 4. The main aim of Experiment 1 was to document any developmental trend in general activity levels in 4 to 7 dpf zebrafish larvae and to determine any age-dependent preferences for spatial location and orientation during this period. In Experiment 2, different groups of larvae from the same egg collection were observed at 4, 5, 6 and 7 dpf. The larvae were placed in 96 micro-well plate. Both experiments found significant behavioral differences in resting and a lesser extent of activity between 4 dpf and older larvae. There was a biased preference for the edge region and an outward facing orientation across all four ages. But there is a possibility that activity in 4 dpf larvae may be more variable than at any other age [30].

In another type of such studies, shoaling behavior has been observed. Here, age-dependent changes of shoaling behavior in freely moving groups of zebrafish have been analyzed. Shoaling is thought to provide the individual fish with multiple benefits including access to mates, efficient foraging and defense against predators. Three experiments were conducted, in which the zebrafish were allowed to explore the glass tank freely. In experiments 1 and 2, the arena size was kept proportional to the body length of the growing fish (for a particular age-group), but in experiment 3, two separate age-groups of fish (30 and 60 day old) were tested in six different arena sizes each.

Experiment 1: Longitudinal developmental analysis of shoaling

The purpose of this experiment was to investigate the trajectory of potential age-dependent changes of shoaling behavior in zebrafish. The same group of fish were followed throughout their development (a repeated measure design). All open field tanks employed for older age groups were scaled-up versions of the smallest tank (90*90*30 mm width*length*depth) used for the 3.2 mm long 5 dpf old fish. It was found that at the youngest age tested zebrafish appeared dispersed though they were attracted (slightly) to each other. The distance among shoal members decreases with age and reaches 5.81 body-lengths at 76 dpf.

Experiment 2: Cross-sectional analysis of age differences in shoaling

Since repeated handling induced elevation in fear could lead to enhanced shoaling, a non-repeated measure cross-sectional experiment was conducted. Shoaling behavior of seven different age groups of fish were analyzed. The fish were tested in the open field only once. The order of testing fish of different ages was randomized. This analysis confirmed that shoal density increased with age.

Experiment 3: Randomization of open field size

Two age groups of fish (30 dpf and 60 dpf) were exposed to different tank sizes but instead of increasing tank sizes over time, exposure to different arena sizes was randomized. It was found out that increasing tank sizes did not lead to decreasing average distance among shoal members in either age group studied [28].

CONCLUSION

Zebrafish model in neuro-behavior research serves as an ideal resource for scientists seeking valuable insight into the growing utility of zebrafish in neuroscience. The larval zebrafish is an excellent model for investigating locomotory kinetics as well as drugs with anxiolytic properties. High-speed video recordings of behavioral responses in this species are indeed very promising for high-throughput screening (which is not feasible in rodent models). In future studies some of the questions like how other neurotransmitter systems may be involved in shoaling in zebrafish and also importantly what neural circuits, and in general which brain areas, may be involved should be pondered upon. Zebrafish can be used for designing models to reverse drug seeking behavior such as punishment or reinforcement associated to withdrawal in order to weaken the brain reward systems. Also studies which show changes in brain because of low and high alcohol doses must be done. Future forward genetic analysis must be done to identify mutations that show altered sensitivity to ethanol that shall provide important insights into the genes involved in regulating the behavior.

CONFLICT OF INTEREST

There is no conflict of interest among authors.

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ARTICLE

FUZZY CONTROL OF INTERNAL COMBUSTION ENGINES

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ABSTRACT

To control a diesel engine in the process of testing, the principles of fuzzy output, which are widely used in fuzzy-logic controller development, could be applied. Controller's main task is to monitor an external object, in which case the behavior of the monitored object is described by the fuzzy rules. The most important application area of fuzzy sets theory is the fuzzy logic controllers. Their operation slightly differs from the operation of common controllers. In order to describe the system, the expert knowledge is used instead of differential equations. Control of the automation systems for engine testing (ATS), using fuzzy-logic controller, should be based on a knowledge database with fuzzy rules. Such database could be created with expert knowledge, neural network, or direct measuring method. Development of an adaptive control system for diesel engine testing process based on fuzzy logic allows to simplify system's structural components and to provide discrete control procedure with some uninterruptible properties, which could improve the control and reduce the scope of the knowledge database. Fuzzy logic makes it fairly simple to input a priori information about an object in the form of fuzzy control rules into the adaptive control system. Similarity of form and natural language relatively easy allows to obtain necessary expert knowledge. A priori information provides one of the key initial conditions of the system developed according to adaptive control method – the condition of supreme initial adaptation.

INTRODUCTION

KEY WORDS
control; network;
diagnostic; system;
engine.

The knowledge database is a crucial component of diesel engine testing intelligence system. The knowledge database is a set of facts and inference rules, supposing logical inference and intelligent data processing [1].

This database consists of three levels.

The first level includes linguistic variables. These variables are put together by the process engineers at the stage of test development with regard to a specific engine model. The variables should include the parameters necessary to setup and control the testing procedures. Linguistic variables are filled in by the experts in this particular area and with the help of direct measuring methods. Basic limits and the number of linguistic variables, together with the type of membership function, should be defined at this level. The number of linguistic variables depends on the control accuracy. However, this will require more time to fill in the knowledge database. This level also contains syntactic and semantic rules for the linguistic variables.

The second level contains fuzzy rules used to convert the given parameters into the control ones. These rules are composed from the linguistic variables defined at the previous level. They can be set by the experts, with the help of direct measuring methods, or by the self-learning neural network. In the latter case, the level will be completed with data fully automatically. This level also includes semantic rules, which define the feasibility of testing. For example, it is impossible to obtain maximum power or torque under given minimum speed. These rules also allow to avoid fault situations.

The third level contains the priority vectors used to range engine characteristics. The priority vectors consist of set parameters. They can be used to focus the tests, i.e. to test ecological properties, power, or economic efficiency of the engine.

The ATS for diesel engines provides a feedback during testing procedures. This feedback allows both to fix the results and adjust the control inputs, and to complete the knowledge database with the help of case study method.

MATERIALS AND METHODS

Case-based inference is a method of decision-making that uses the knowledge about previously occurred situations or accidents (cases). When considering a new problem (current situation), a similar case should be found to be used as a precedent. It is possible to use a solution developed for a similar case, adjusting it to the new conditions of the current case, if necessary, instead of searching a solution for a new case each time from the very beginning. After the current case has been processed, it can be introduced into the case database together with its solution for possible further application [2].

A case includes:

- problem description;
- problem solution;
- result (validity) of solution application.

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Problem description should contain all information necessary to achieve the goal of the inference (the selection of the most relevant solution). In the ATS for diesel engines, problem description represents the types of engine operating conditions.

Result description can contain information about positioning of controls at a certain point, execution results, restoring method (in case of failure), description of operations to be performed in order to avoid the failure, restoring results. Result description can also include the references to other cases, or additional text information.

A case may contain not only the positive results. It is necessary to save information about the failures of application of some specific operating conditions in order to avoid their further usage. Descriptions of failures and their causes can be used in future. The system can store explanations of a decision and even its alternatives [3].

Case study method includes the following stages:

- extraction of the most relevant cases for the current situation from the case database;
- adjustment of the selected solution to the current situation, if necessary;
- solution application;
- application evaluation (validity check);
- adding and saving of current case into the case database.

Let's consider database completion under dynamic conditions of diesel engine testing as an example [4]. In this case a fuzzy rule will be as follows:

IF ω_i AND R_j THEN h_i

Where

ω_i – is the crankshaft (engine) speed ($\omega_1, \omega_2, \omega_3, \dots, \omega_n$);
 R_j – is the engine operating condition ($R-R; \dots R-1; R_0; R_1, \dots; R_r$);
 h_i – is the rack displacement (h_1, h_2, \dots, h_n).

These rules can be used to create the database for steady-state engine operating conditions. In this case the level R_j will have a fixed value of R_0 . This means that the engine is working under a condition, when the speed is neither increasing, nor decreasing [5]. The database of the steady-state conditions will be defined as follows:

IF ω_i AND R_0 THEN h_k

where

ω_i – is the engine speed ($\omega_1, \omega_2, \omega_3, \dots, \omega_n$),
 R_0 – is the steady-state engine operating condition,
 h_k – is the rack displacement (h_1, h_2, \dots, h_l).

The level R_j is completed with the help of case study method. Let's assume that we have the control rule:

IF ω_k AND R_1 THEN h_x

where

ω_k, R_1 – are the fixed linguistic variables of the variables ω_i and R_j ,

h_x – is the unknown value of the variable h_k .

Let's find the nearest case to complete this rule. The nearest rule is the following:

IF ω_k AND R_0 THEN h_s

where

ω_k, R_0, h_s – are fixed linguistic variables of variables ω_i, R_j and h_k .

Let's create two new fuzzy rules based on this case:

IF ω_k AND R_1 THEN h_s
 IF ω_k AND R_1 THEN h_{s+1}

Control value of the rule 1 coincides with the nearest case, while for the rule 2 the nearest linguistic variable located towards mutable variable R_j should be taken (if the variable R_j of a new rule is higher, than that of the case, then the control value shifts upwards towards the value of the nearest linguistic variable, otherwise – downwards).

The result of the first rule application is known, as it fully coincides with the case. The result of the second rule should be checked under the self-learning mode. If the result of the first rule is more accurate, then it should be used as a basis and the database completion comes to an end. Otherwise, the rule 2 becomes the rule 1, while the rule 2 should be developed again on the basis of the next linguistic variable:

IF ω_k AND R1 THEN $hs+1$
 IF ω_k AND R1 THEN $hs+2$

Both rules should be tested for accuracy, and, if the rule 1 more accurate, then it should also be taken as a basis. Otherwise, the replacement, similar to that mentioned above, should be performed.

If the most accurate rule doesn't meet the required accuracy, then an intermediate linguistic variable should be introduced, using intersection operation [6]. If the assignment of the basic value to two nearest variables is equally possible, then a new linguistic variable should be created there.

Introduction of a new linguistic variable will make it possible to develop the following control rule:

IF ω_k AND R1 THEN $hs+1, s+2$

If this rule meets the required accuracy, then it is retained in the database [7]. Otherwise, a new linguistic variable should be created within the limits of two linguistic variables with the smallest measure of inaccuracy.

The number of linguistic variables is limited by the digit capacity of the fuzzy controller. Increased number of linguistic variables can affect real-time operation mode.

Application of this method will allow to reduce time required for knowledge database creation and to improve the accuracy of control, while increasing the number of tests [8]. Case study method application also allows to adjust the knowledge database created for one engine to another engine with slightly different characteristics. Time required to create the database will depend on how the operation conditions differ.

RESULTS AND DISCUSSION

The process of development of the ATS for diesel engines on the basis of control flow chart with fuzzy controller application represents the following sequence of stages [9].

The upper level represents the testing procedure, namely the document, regulating the performance of all types of diesel engine tests.

It contains the key aspects of the tests: purpose and goal, scope, condition for selection of a test object, testing equipment, testing conditions and testing procedure, result estimation methods, technical and fire safety requirements, industrial health requirements [10]. Testing procedure is developed by the process engineer, who takes into account all necessary engine parameters [11]. While developing the procedure, the process engineer should specify engine characteristics, which he would like to obtain as the output result [12]. The ATS for diesel engines will automatically select optimal control parameters to execute specified modes.

At the next stage the above-mentioned testing procedure is represented in the form of patterns [13]. A pattern is a sequence of changing modes, each of which could be represented as a segment, which X-component corresponds to mode run-time, while Y-component characterizes the changes in a measured parameter for that time period.

While the graphical representation is more convenient to a human being, a machine uses numeric data for processing [14]. Therefore, the conversion of the patterns into a summary table of parameters should be performed at the next stage [15, 16, 17, 18, 19]. For this purpose, test time should be divided into the time intervals t_{min} , at which engine operation control will be executed. A time interval should be as small as possible to provide improved control accuracy and to avoid fault situations. However, reduced time intervals require significant computing facilities. The number of points, at which the control will be executed during the testing procedure, is defined by ratio:

$$S = t_{is} / t_{min}$$

where

S – is the number of points;

t_{is} – is total test time;

t_{min} – a time interval between two control points.

Further, the values of measured parameters ($A_{1i}, A_{2i}, A_{3i}, \dots, A_{mi}$) should be assigned to each time interval, where A_i is the parameter values at a certain point derived from respective patterns. This data is saved in the summary table of parameters.

It is worthwhile to apply fuzzy logic to the objects characterized by a large number of uncertainties, for example, in case of inaccurate information received from the actuators, or inaccuracy of the model of the monitored objects and engine control [21, 22, 23, 24]. The results of control will be considered appropriate, if they fall within the corresponding limits [25]. Fuzzy logic application with regard to diesel engine testing procedure control allows to assess aggregate impact of each parameter on the final computation result, while the ignorance of the minor parameters by the other methods leads to erroneous outcome.

CONCLUSIONS

The above methods are characterized by one essential fault, lying in the fact that all parameters included into the fuzzy rules equally influence on the output characteristic. As a result, it is impossible to develop the focused tests. For example, when performing the fuel consumption test, fuel consumption parameter will be of primary importance. This parameter should be also considered in wearing test, though it will be less significant. To avoid this shortcoming, it is necessary to develop a system, controlling the impact of each specific parameter on the final result.

CONFLICT OF INTEREST

There is no conflict of interest.

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ARTICLE

A NEW APPROACH FOR THE DETERMINATION OF SPECIES SAPROBITY FOR WATER QUALITY MONITORING BASED ON THE MOLECULAR PHYLOGENY

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ABSTRACT

The well-known method of bioindication is widely used for water quality monitoring. The number of indicator species is low in a compare with total number of species. For example, the indicator list of freshwater Bacillariophyceae made by V. Sladeczek includes only 192 species of organisms from estimated 100000 species. The same situation happens with other freshwater organisms, which have not the status of indicator until nowadays. For solving this problem, we suggest a new approach for determination of indicator species for water quality monitoring based on the molecular phylogeny. Our choice is the *rbcL* gene and product of *rbcL* gene – protein of Bacillariophyceae, which is used as marker gene for plants. Phylogenetic analysis includes 66 sequences of *rbcL* gene and *rbcL* protein of Bacillariophyceae bioindicators accordingly from GenBank and GenPept Sequences Databases on NCBI website. As results, two phylogenetic trees on *rbcL* gene and two trees on *rbcL* protein of Bacillariophyceae were constructed using Neighbor-Joining (NJ) and Maximum Parsimony (MP) methods with bootstrap. The comparative analysis of phylogenetic trees shows stable clustering of the indicator species of different genera with the same or close saprobity with higher bootstrap by the *rbcL* gene than by the *rbcL* protein. Obtained results allow us to find new bioindicators faster than by using the traditional technology, using less time and resources. Thus, we conclude that our new technology can be used for the water quality monitoring and research results have the fundamental and practical value.

INTRODUCTION

Water quality is a complex subject, because water is a complex medium intrinsically tied to the ecology of the Earth and it shows large geographical variation. The most common standards used to assess water quality relate to health of ecosystems, safety of human exposure, and drinking water quality. Water quality refers to the chemical, physical, and biological characteristics of water. Biological monitoring of water quality is based on bioindicators. A bioindicator is any species whose function, population, or status reveal the qualitative status of the environment. We used plankton, because it is geographically independent, it is convenient for detection and it quickly reacts to pollutants of any type. For example, members of plankton such as microalgae.

We work with phytoplankton samples of diatoms or *Bacillariophyceae*, which are a major group of micro-algae, and the most common types of phytoplankton. Diatoms with other water inhabitants are used as saprobity indicator species in the monitoring of the water quality. Traditional technology for bioindicator identification include sample collection, identification of species by morphological characteristics using the microscope and construction of the bioindicators list. There are only 192 known indicator species of diatoms from list of Sladeczek (1973) [1]. That is few as compared with more than 200 genera with an estimated 100000 species. The same situation is happening with other organisms, which have not the status of indicators until nowadays. This is explained by the long-time used in analysis and high cost of the experimental work with living organisms. To reduce costs and increase productivity we suggest the alternative approach of bioindicators determination, building on the existing database. It includes the sequences collection from free international database and analysis of molecular phylogeny [2]. Our choice is the gene and product of *rbcL* *Bacillariophyceae* which is used as marker gene for plants [3, 4]. We used the *rbcL* gene of fresh water plants for the monitoring of Kaban Lakes [5, 6]. Our aim is to development of more efficient methods for bioindication based on modern methods of bioinformatics and molecular genetics for water quality monitoring.

MATERIALS AND METHODS

Phylogeny analysis includes sequences of *rbcL* gene and *rbcL* protein of *Bacillariophyceae* – fresh water indicator species given by Sladeczek's list and species from Kaban Lakes (Kazan, Russia). The *rbcL* sequences of 66 indicator species of *Bacillariophyceae* were performed in international databases GenBank Nucleotide and GenPept Protein Sequences on NCBI website (www.ncbi.nlm.nih.gov).

Multiple alignments of nucleotide and protein of *rbcL* sequences of indicator species were made with Clustal Omega program (<https://www.ebi.ac.uk/Tools/msa/clustalo/>) [7]. Molecular phylogenetic trees by Neighbor-Joining (NJ) method [8-11] and Maximum Parsimony method (MP) [9, 11, 12] were constructed using the MEGA program (www.megasoftware.net) [13].

KEY WORDS

saprobity,
determination, water
quality, DNA/protein
rbcL, Bacillariophyceae,
bioindicators

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[Table 1] shows the indicator species of *Bacillariophyceae* from the Sladeczek's list and from Kaban Lakes with their saprobity and accession numbers from GenBank and GenPept databases. The new species name, if renamed, shown in parentheses.

Table 1: Accession numbers obtained from GenBank and GenPept databases of *rbcL* Bacillariophyceae species with the saprobity

Species	Saprobity*	<i>rbcL</i> gene GenBank	<i>rbcL</i> protein GenPept	Species of Kaban lakes
<i>Achnanthydium coarctatum</i>	x	HQ912458	AEB91214	-
<i>Achnanthes minutissima</i> (<i>Achnanthydium minutissimum</i>)	o-b	KY863484	ASK50010	-
<i>Amphora ovalis</i>	b-o	KC954577	AHK61273	+
<i>Amphora pediculus</i>	-	HQ912403	AEB39384	+
<i>Anomoeoneis sphaerophora</i>	b-o	KJ011795	AIY31906	-
<i>Asterionella granulata</i> (<i>A. formosa</i>)	o-b	HQ912497	AEB91253	+
<i>Aulacoseira granulata</i>	b-o	KM999116	AKS44355	+
<i>Caloneis amphisbaena</i>	b-a	KM084980	AIT92080	-
<i>Caloneis silicula</i>	o-b	JN418663	AER42044	-
<i>Cocconeis pediculus</i>	b-o	KM084977	AIT92077	-
<i>Cocconeis placentula</i>	o-b	HQ912456	AEB91212	+
<i>Cocconeis placentula</i> var. <i>euglypta</i>	-	KT072907	ALO24317	+
<i>Cyclotella bodanica</i>	o	DQ514829	ABF60387	+
<i>Cymatopleura elliptica</i>	b	KX120659	AQM56457	-
<i>Cymatopleura solea</i>	b-a	KX120661	AQM56459	-
<i>Cymbella affinis</i>	b-o	KJ011796	AIY31907	-
<i>Cymbella aspera</i>	b	KJ011798	AIY31909	-
<i>Cymbella cistula</i>	b-o	KJ011802	AIY31913	-
<i>Cymbella granulata</i> (<i>C. helvetica</i>)	x-o	KJ011804	AIY31915	-
<i>Cymbella lanceolata</i> (<i>Brebissonia lanceolata</i>)	b	KJ011806	AIY31917	-
<i>Cymbella naviculiformis</i> (<i>Cymbopleura naviculiformis</i>)	b	KJ011815	AIY31926	-
<i>Didymosphenia granulata</i> (<i>D. geminata</i>)	x	KJ011819	AIY31930	-
<i>Encyonema ventricosum</i>	o-b	KU052340	ALN50604	+
<i>Epithemia sorex</i>	b	HQ912395	AEB39376	-
<i>Epithemia turgida</i>	b	KX120566	AQM56364	-
<i>Eunotia pectinalis</i>	x	HQ912500	AEB91256	-
<i>Fallacia pygmaea</i>	a	HQ912469	AEB91225	-
<i>Fragilaria capucina</i>	b-o	KC736594	AGG86633	+
<i>Fragilaria crotonensis</i>	o-b	KF959640	AHE78119	+
<i>Gomphonema acuminatum</i>	b-o	KJ011853	AIY31964	-
<i>Gomphonema angustatum</i>	o	KJ011835	AIY31946	-
<i>Gomphonema capitatum</i>	b	AY571751	AAT78574	-
<i>Gomphonema clevei</i>	x	JQ354682	AFV95053	-
<i>Gomphonema intricatum</i>	o-x	KJ011840	AIY31951	-
<i>Gomphonema intricatum</i> v. <i>pumilum</i> (<i>G. pumilum</i>)	o	KC736599	AGG86638	-
<i>Gomphonema parvulum</i>	b	JQ354693	AFV95052	-
<i>Gomphonema truncatum</i>	-	AM710509	CAM97966	+
<i>Gyrosigma acuminatum</i>	b	KM999078	AKS44317	-
<i>Melosira granulata</i> var. <i>angustissima</i> (<i>Aulacoseira granulata</i> v. <i>angustissima</i>)	b-o	FJ002130	ACS92840	-
<i>Melosira varians</i>	b-o	KM999081	AKS44320	-
<i>Navicula cryptocephala</i>	a	HQ912467	AEB91223	+
<i>Navicula tripunctata</i>	b-o	KM084935	AIT92035	-

Species	Saprobity*	rbcl gene GenBank	rbcl protein GenPept	Species of Kaban lakes
<i>Navicula gregaria</i>	b	KY320297	ASC55339	-
<i>Navicula radiosa</i>	b-o	KM084955	AIT92055	-
<i>Nitzschia acicularis</i>	a	KX889095	ASF62417	+
<i>Nitzschia dissipata</i>	o-b	KY320333	ASC55375	-
<i>Nitzschia fonticola</i>	o-b	HF675068	CCQ77735	-
<i>Nitzschia linearis</i>	o-b	KT072917	ALO24327	+
<i>Nitzschia palea</i>	a	FN557017	CBH19895	-
<i>Nitzschia sigmaidea</i>	b	FN557033	CBH19911	-
<i>Pinnularia borealis</i>	x-o	JN418640	AER42021	-
<i>Pinnularia viridis</i>	b	KM350021	AKH66073	-
<i>Rhoicosphenia abbreviata</i>	b-o	KJ011854	AIY31965	-
<i>Rhopalodia gibba</i>	o	KX120556	AQM56354	-
<i>Stauroneis acuta</i>	o	HQ912443	AEB91199	-
<i>Stauroneis anceps</i>	b	AM710475	CAM97934	-
<i>Stauroneis phoenicenteron</i>	b-o	KM084992	AIT92092	-
<i>Stephanodiscus hantzschii</i>	a	AB831882	BAV19460	+
<i>Surirella biseriata</i>	b	JX033009	AGE34629	-
<i>Surirella capronii</i>	b	JX033000	AGE34620	-
<i>Surirella splendida</i>	b-o	HQ912401	AEB39382	-
<i>Surirella spiralis</i>	o	JX032964	AGE34584	-
<i>Surirella tenera</i>	b	JX033012	AGE34632	-
<i>Synedra ulna (Ulnaria ulna)</i>	b	HQ912454	AEB91210	+
<i>Tabellaria flocculosa</i>	o-x	HQ912448	AEB91204	+
<i>Ulnaria delicatissima var. angustissima</i>	o	KT072900	ALO24310	-

* x- (pure), o-(clean), b- (polluted), a- (very polluted)

RESULTS AND DISCUSSION

As known, the traditional technology needs of experimental work with living organisms. As a result, it takes a long time before the specie can be determined as a bioindicator. In a compare, our innovative approach uses modern methods of bioinformatics and molecular phylogenetics, which allow us to determine new bioindicators faster than by using the traditional technology, using less time and resources.

Thus, the new approach includes the selection of 66 primary sequences of rbcl gene and rbcl protein of *Bacillariophyceae* bioindicators from international databases; multiple alignment of all sequences; construction of phylogenetic trees by rbcl gene and rbcl protein of *Bacillariophyceae* bio indicators using computers.

As a result, two phylogenetic trees on rbcl gene [Fig. 1] and two phylogenetic trees on rbcl protein [Fig. 2] of 66 indicator species of *Bacillariophyceae* were constructed by NJ- and MP-methods with bootstrap. The percentage of bootstrap from 100 replicas for NJ/MP trees are shown accordingly next to the nodes.

Phylogenetic analysis of rbcl gene of bacillariophyceae

As can be seen from [Fig. 1], there are 17 clusters on the phylogenetic tree with high bootstrap more than 50%, three of them include non-indicators species:

- cluster 1 includes species from the same genus – *Surirella* mainly of b-saprobity;
- cluster 2 includes species from the same genus – *Cymatopleura* mainly of b-saprobity;
- cluster 3 includes species from different genera – *Rhopalodia* and *Epithemia* mainly of b-saprobity;
- cluster 4 includes species from the same genus – *Aulacoseira* of b-o-saprobity;
- cluster 5 includes species from different genera – *Fallacia* and *Pinnularia* of different-saprobity;
- cluster 6 includes species from the same genus – *Stauroneis* mainly of b-o-saprobity;
- cluster 7 includes species from different genera – *Ulnaria*, *Synedra* and *Fragilaria* mainly of o-b-saprobity;
- cluster 8 includes species from the same genus – *Nitzschia* of a-saprobity;

- cluster 9 includes non-indicator species – *Amphora pediculus* and indicator species – *Amphora ovalis* of b-o saprobity;
- cluster 10 includes species from the same genus – *Navicula* of different-saprobity;
- cluster 11 includes non-indicator species – *Cocconeis placentula* var. *euglypta*, indicator species from different genera – *Cocconeis* and *Rhoicosphenia* of b-o-saprobity and species from different genera – *Cocconeis* and *Achnantheidium* of o-b- saprobity;
- cluster 12 includes species from the same genus – *Gomphonema* of different-saprobity;
- cluster 13 includes non-indicator species – *Gomphonema truncatum* and species from the same genus – *Gomphonema* mainly of b-o-saprobity;
- cluster 14 includes species from different genera – *Cymbella* and *Cymbopleura* of different-saprobity;
- cluster 15 includes species from different genera – *Asterionella* and *Tabellaria* mainly of o-saprobity;
- cluster 16 includes species from different genera – *Cyclotella* and *Stephanodiscus* of different-saprobity;
- cluster 17 includes species from the same genus – *Nitzschia* mainly of b-saprobity.

As we can see from [Fig. 1], all organisms are grouped in the clusters with the same and/or close saprobity with a high bootstrap. For example, cluster 8 includes the species *Nitzschia acicularis* and *Nitzschia palea* of a-saprobity with a high bootstrap more than 95%. This is a very good result.

Phylogenetic analysis of rbcL protein of *bacillariophyceae*

As can be seen from [Fig. 2], in a compare with phylogenetic tree on rbcL gene of *Bacillariophyceae* there are only 12 clusters with high bootstrap more than >50%, three of them include non-indicator species:

- cluster 1 includes species from different genera – *Navicula* and *Gyrosigma* mainly of b-o-saprobity;
- cluster 2 includes species from the same genus – *Nitzschia* mainly of a-saprobity;
- cluster 3 includes species from the same genus – *Nitzschia* mainly of o-b-saprobity and species from different genera – *Stephanodiscus* and *Cyclotella* of different-saprobity;
- cluster 4 includes species from different genera – *Ulnaria*, *Synedra* and *Fragilaria* mainly of o-b-saprobity;
- cluster 5 includes species from the same genus – *Aulacoseira* of b-o-saprobity;
- cluster 6 includes species from the same genus – *Surirella* mainly of b-saprobity;
- cluster 7 includes species from different genera – *Cymatopleura* and *Amphora* mainly of b-saprobity;
- cluster 8 includes species from the same genus – *Epithemia* of b-saprobity;
- cluster 9 includes non-indicator species – *Amphora pediculus* and species from different genera – *Rhopalodia* and *Surirella* of o-saprobity;
- cluster 10 includes species from the same genus – *Gomphonema* of different-saprobity;
- cluster 11 includes non-indicator species – *Cocconeis placentula* var. *euglypta*, and species from the same genus – *Cocconeis* mainly of b-o-saprobity;
- cluster 12 includes non-indicator species – *Gomphonema truncatum*, and species from the same genus – *Gomphonema* mainly of b-o-saprobity.

In a comparison of the rbcL protein tree [Fig. 2] with the rbcL gene tree [Fig. 1], we can see that less species grouped in clusters with high bootstrap. We may make the conclusion that the comparative analysis of phylogenetic trees shows stable clustering of indicator species with the same or close saprobity with higher bootstrap by the rbcL gene than by the rbcL protein.

Checking assessment results

We need to check the preliminary assessment results using data from natural lakes, for example the plankton species from the big Kaban Lakes in Kazan, which are situated in the center of the city. The Kaban Lakes are a system of lakes, which includes Nizhny Kaban, Verkhny Kaban, and Sredny Kaban. With a combined area of 1.86 square kilometers, they comprise the biggest lake in Tatarstan Republic (Russia). The ecologists appreciate the lakes as the transition from polluted (b-saprobity) to pure (o-saprobity).

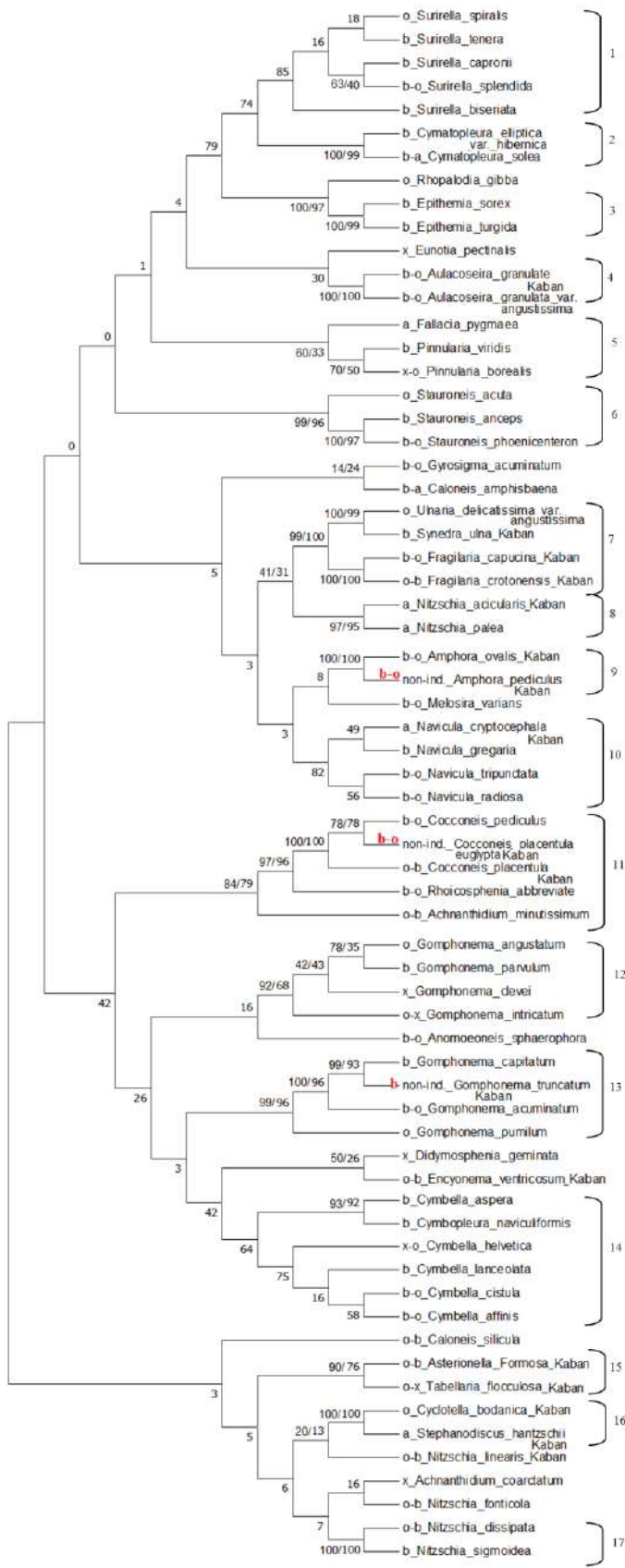


Fig. 1: Phylogenetic tree on gene of rbcL Bacillariophyceae (NJ/MP methods).

Molecular phylogenetic analysis includes indicator species and non-indicator species of *Bacillariophyceae* from Kaban Lakes. For non-indicator organisms, the saprobity can be determined based on phylogenetic analysis [Fig. 1]:

- cluster 9 with bootstrap equal 100% includes indicator species *Amphora ovalis* of o-saprobity and non-indicator species - *Amphora pediculus*, that means the last one should be the same o-saprobity [Fig. 3];

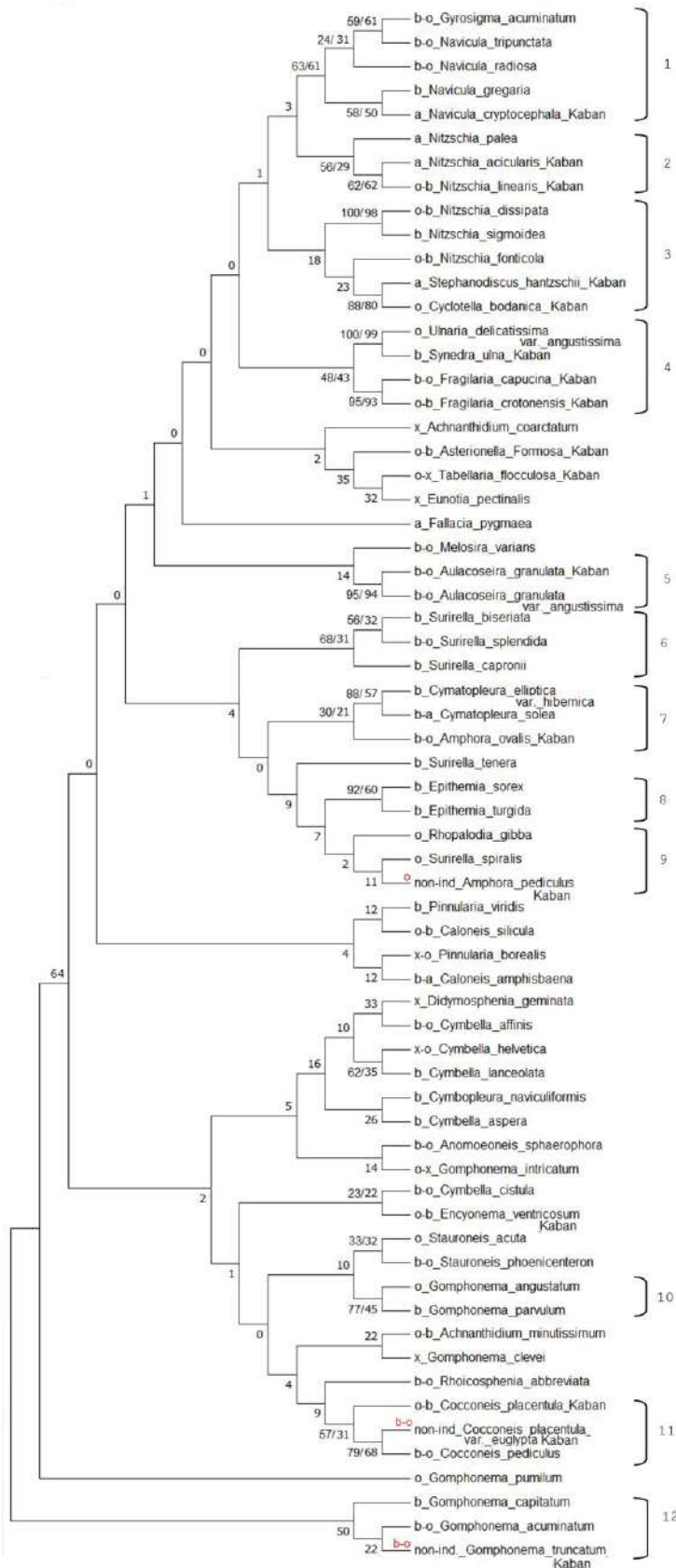


Fig. 2: Phylogenetic tree on protein of rbcL Bacillariophyceae (NJ/MP methods).

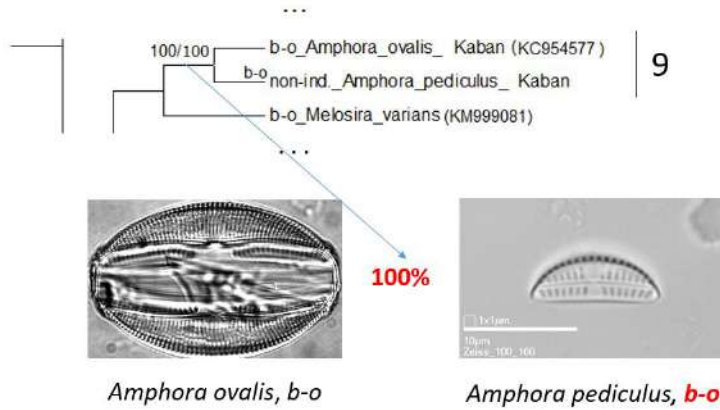


Fig. 3: The fragment on gene *rbcl* Bacillariophyceae with the cluster 9.

- cluster 11 with bootstrap equal 78% includes indicator species *Cocconeis pediculus* of b-o-saprobity and non-indicator species - *Cocconeis placentula* var. *euglypta*, that means the last one should be the same b-o-saprobity [Fig. 4];

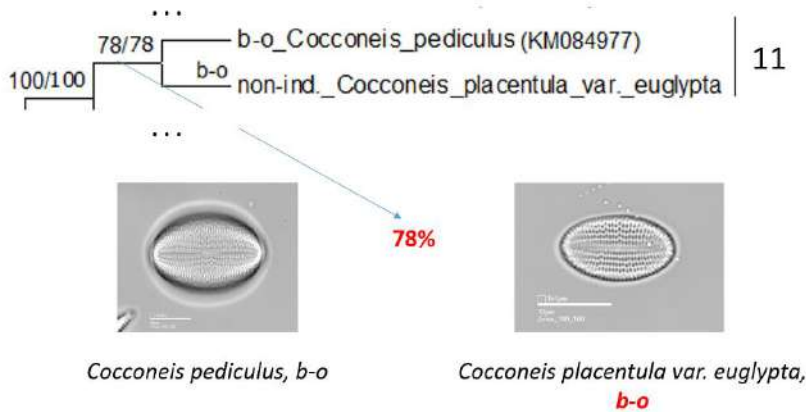


Fig. 4: The fragment on gene *rbcl* Bacillariophyceae with the cluster 11.

- cluster 13 with high bootstrap equal 99% includes indicator species *Gomphonema capitatum* of b-saprobity and non-indicator species - *Gomphonema truncatum*; that means the last one should be the same b-saprobity with high bootstrap [Fig. 5].

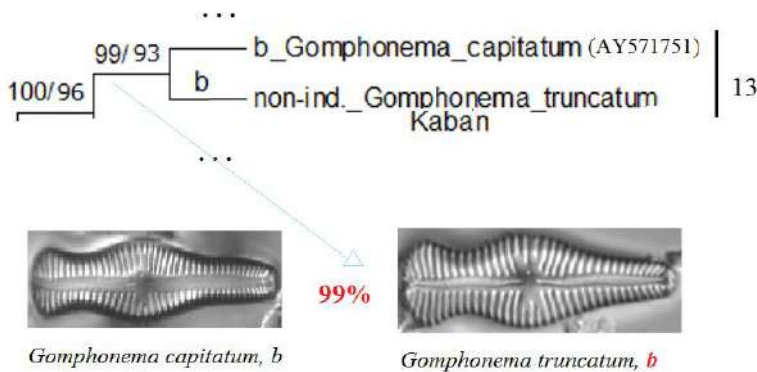


Fig. 5: The fragment on gene *rbcl* Bacillariophyceae with the cluster 13.

As a result of the phylogenetic analysis we have a new list of *Bacillariophyceae* bio indicators for the Kaban Lakes with additional bio indicators: *Amphora pediculus*, *Cocconeis placentula* var. *euglypta* and *Gomphonema truncatum* [Table 2].

Table 2: The list of *Bacillariophyceae* bioindicators for the Kaban Lakes with additional bioindicators species

Species	Saprobity	rbcl gene	rbcl protein
<i>Tabellaria flocculosa</i>	o-x	HQ912448	AEB91204
<i>Nitzschia linearis</i>	o-b	KT072917	ALO24327
<i>Cyclotella bodanica</i>	o	DQ514829	ABF60387
<i>Amphora ovalis</i>	b-o	KC954577	AHK61273
<i>Melosira granulata</i> (<i>Aulacoseira granulata</i>)	b-o	KM999116	AKS44355
<i>Synedra ulna</i> (<i>Ulnaria ulna</i>)	b	HQ912454	AEB91210
<i>Navicula cryptocephala</i>	a	HQ912467	AEB91223
<i>Nitzschia acicularis</i>	a	KX889095	ASF62417
<i>Stephanodiscus hantzschii</i>	a	AB831882	BAV19460
<i>Amphora pediculus</i>	b-o	HQ912403	AEB39384
<i>Cocconeis placentula v. euglypta</i>	b-o	KT072907	ALO24317
<i>Gomphonema truncatum</i>	b	AM710509	CAM97966

In case of gene sequences absent in international database, it is easy to get the experimental sequences from water samples.

CONCLUSIONS

Recent results allow us to conclude that a new technology will make water quality monitoring and assessment more efficient. Thus, the new method can be used for effective determination of bio indicators. The comparative analysis of the phylogenetic trees shows stable clustering of indicator species with the same and/or close saprobity with higher bootstrap by the rbcl gene than by the rbcl protein. The technology allows us to determine the saprobity for non-bio indicators based on phylogenetic analysis and add new bio indicators in the total list in an easier way than by experimental work.

CONFLICT OF INTEREST

There is no conflict of interest.

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FINANCIAL DISCLOSURE

None.

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