01 Sep 2002

Introducing Molecular Biology to Environmental Engineers: Through Development of a New Course

Daniel B. Oerther

Missouri University of Science and Technology, oertherd@mst.edu

INTRODUCING MOLECULAR BIOLOGY TO ENVIRONMENTAL ENGINEERS

Through Development of a New Course

DANIEL B. OERTHER
University of Cincinnati • Cincinnati, OH 45221-0071

Historically, applications of biology in chemical and environmental engineering have been approached from different perspectives with different goals. For example, chemical engineering optimizes biochemical reactions of pure cultures of microorganisms in highly controlled bioreactors used for manufacturing (*e.g.,* fermentation), whereas environmental engineering employs mixed microbial communities with minimum controls as least-cost processes for meeting regulatory requirements (*e.g.,* sewage treatment). Although chemical and environmental engineering education often incorporates formal training in biology, the motivation for course selection can be very different. Incremental advances in biological knowledge that can be used to increase manufacturing capability or improve efficiency are useful in chemical engineering practice, and their integration into chemical engineering education is justified.

The same principle does not hold for environmental engineering, however. Once minimum regulatory requirements are met, incremental advances in biological knowledge do not offer the significant cost savings for environmental biological unit operations that are needed to encourage the adoption and integration of the new knowledge into environmental engineering education.

Recently, development of 16S ribosomal ribonucleic acid (16S rRNA)-targeted technology provided researchers in environmental engineering with new tools to identify micoorganisms and to study micoorganisms in bioreactor environments. As compared to classical techniques for identification and enumeration, 16S rRNA-targeted technology allows in situ examination of the structure (*i.e.,* who is present?) and function (*i.e.,* what are they doing?) of microbial communities without a prerequisite for isolating pure cultures.[1] For researchers in environmental engineering, 16S rRNA-targeted technology has been extensively tested, and current research activities have moved beyond the "proof-of-concept" state to widespread applications.[2,3] In contrast, integration of 16S rRNA-targeted technology within the environmental engineering curriculum remains to be fully developed. At the University of Cincinnati, the author has developed and pilot tested a "proof-of-concept" course titled "Molecular Methods in Environmental Engineering."

The course was designed to teach limited fundamentals of molecular biology in the context of quantitative engineering design and practice. During its first offering, fifteen graduate students in environmental engineering were exposed to "state-of-the-art" technology, including hands-on laboratory exercises following the "full-cycle 16S rRNA approach."[1] Students learned the importance of detailed understanding of microbial communities and microbial-mediated biochemical networks in biological unit operations, natural biological systems, and the global biosphere. The format of the course included a weekly lecture as well as a semester-long series of hands-on laboratory exercises designed to teach students to develop scientific questions, learn appropriate methodology, conduct careful experimentation, analyze data, and draw conclusions worthy of presentation to peers. Thus the final outcome of the course included preparation of peer-review quality manuscripts by each team of students as well as one-on-one interviews with the instructor.

FULL-CYCLE 16S rRNA APPROACH

Traditionally, the identification of microorganisms in environmental samples has relied upon semi-selective culturing or direct microscopic examination. These techniques have led to a rudimentary understanding of the role of microorganisms in the global biosphere as well as the importance of microorganisms in public health and biocatalysis. Recently, the techniques for determinative microbiology have been dramatically expanded to include cultivation-and-morphologic-independent identification and enumeration of microorgan-

Daniel B. Oerther *joined the Department of Civil and Environmental Engineering at the University of Cincinnati in 2000. For ten years, he has been adapting methods from molecular biology to identify, enumerate, and measure the physiology of microorganisms in biotechnology processes including wastewater treatment and bioremediation. His research links the results of novel molecular biology assays with mechanistic modeling of bioreactor performance.*

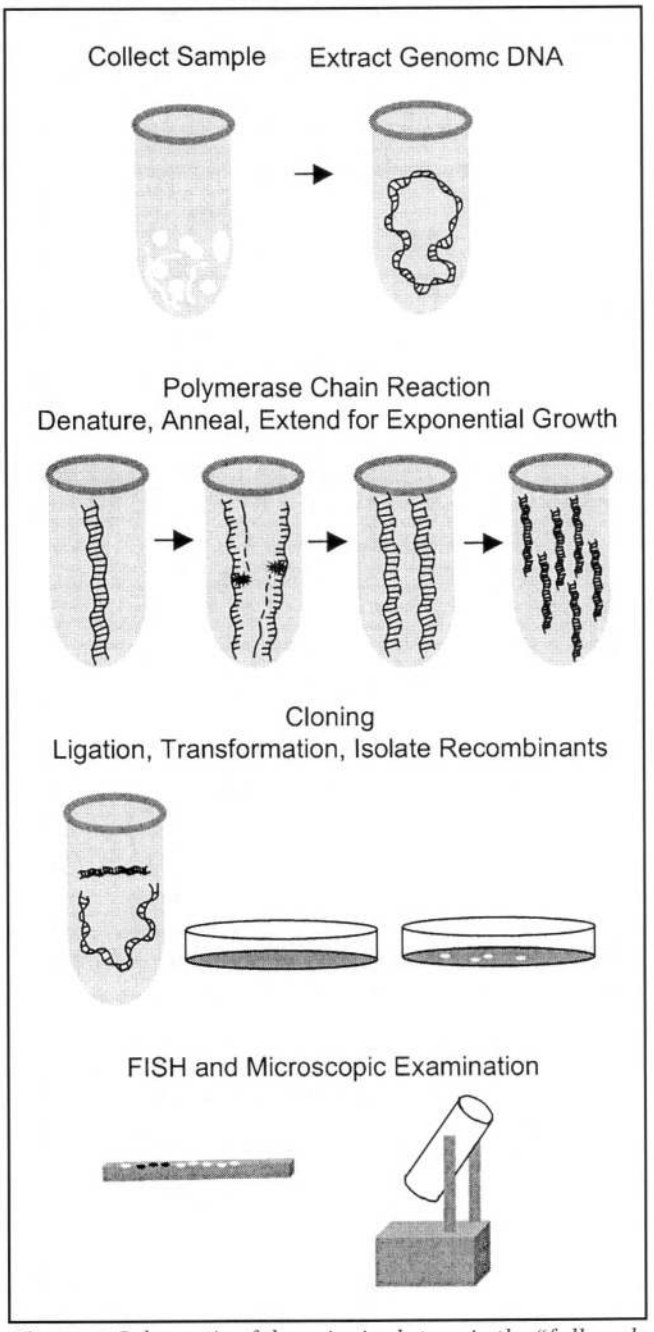

Figure 1. *Schematic of the principal steps in the "full-cycle 16S rRNA approach." Genetic material is isolated directly from an environmental sample and the 16S rDNA genes are amplified in a PCR. The product of the PCR is cloned, and recombinants are isolated for extraction of plasmid DNA. Automated sequencing is used to provide the primary nucleotide structure of the clones, and probe design is accomplished using semi-automated procedures and readily available software. Finally, individual microbial cells are visualized through fluorescence in situ hybridization (FISH) with fluorescently labeled 16S rRNA-targeted oligonucleotide probes.*

isms in environmental samples. Arguably, one of the most widespread families of new techniques for determinative microbiology targets rRNA. Comparative studies of rRNA nucleotide sequences collected from a variety of microorganisms led to the development of a universal phylogenetic framework for understanding the evolutionary history of microorganisms.[4,5] Subsequently, these comparative approaches were coupled with oligonucleotide probe hybridizations to study microorganisms in situ without prerequisite culturing.[1,6]

The "full-cycle 16S rRNA approach" refers to the process of obtaining genomic information directly from an environmental sample and then employing molecular methods to assay the abundance of nucleotide sequences directly within an environmental sample. The steps of the cycle, as applied in my course, are briefly described and outlined in Figure 1. Genomic deoxyribonucleic acid (DNA) is extracted from an environmental sample using chemical and physical disruption of the microorganisms. Subsequently, a polymerase chain reaction (PCR) is used to selectively "grow-up" target genes from the heterogeneous pool of genetic material. In our case, the target genes are 16S rRNA. The target genes, amplified in the PCR, are cloned into bacterial vectors and transformed into competent cells of *Escherichia coli*. The recombinant clones are cultured and plasmid DNA is extracted. The results from commercial dideoxy terminal sequencing are used to design an oligonucleotide hybridization probe purchased from a commercial vendor. The fluorescently labeled probe is hybridized to a "fixed" sample, and individual microbial cells are identified using an epifluorescence microscope.

For my class, commercially available kits were used to the extent possible to minimize the time spent by students and the teaching assistant in preparing reagents. Genomic DNA was extracted using an UltraClean Soil DNA Isolation Kit.[7] PCR was conducted using a model 2400 thermal cycler[8] and the Takara Ex Taq kit.[9] Cloning of the PCR products was accomplished with the TOPO TA Cloning kit version K2,[10] and plasmid DNA was prepared using PerfectPrep Plasmid Mini preps.[11] Throughout the exercises a variety of equipment was used including an ultra low temperature freezer,[12] a Mini Beadbeater-8,[13] a system for agarose gel electrophoresis,[14] a Genesys 10uv,[15] a constant-temperature rotary shaker,[16] and an epifluorescence microscope.[17]

FORMAT FOR LABORATORY EXERCISES

Step 1 • Students arranged themselves into teams of three. The selection of teammates was based both on a common interest in one environmental sample and on an effort to spread previous experience and expertise in molecular biology among the groups.

Step 2 • Teams identified, evaluated, and proposed an appropriate environmental system for study. Each system se-

. . . we plan to expand the enrollment [in this course] to include undergraduate environmental engineering students as well as graduate and undergraduate students from related disciplines, including chemical engineering and biomedical engineering.

lected for the course was novel for the field of environmental engineering and possessed the capacity to stimulate a more extensive research question (*e.g.,* supplemented a research question in an existing/developing MS or PhD degree, or promoted a novel research direction generally underexplored.) A sample was obtained from the selected system. In all cases, preference was placed on samples that were a part of a developing/ongoing research project with significant supplementary information generated from advanced process engineering and chemical/physical analyses (*e.g.,* sample(s) from a novel bioreactor configuration or a bioreactor treating a novel waste stream).

Step 3 • Each team generated 16S rDNA sequence information from their sample(s). Genomic DNA was extracted using an UltraClean Soil DNA Isolation Kit[7] according to the manufacturer's instructions. Mechanical lysis of the samples was performed for one minute at the maximum setting of a Mini Beadbeater-8.[13] Genomic DNA was quantified using a Genesys 10uv[15] spectrophotometer assuming that an absorbance reading of 1.0 at a wavelength of 260 nm corresponded to a concentration of 50 mg DNA/l.

The 16S rDNA genes of bacteria present in the sample were amplified by PCR using primer set S-D-Bact-0011-a-S-17 (5' to 3' sequence = gTT TgA TCC Tgg CTC Ag) and S-D-Bact-1492-a-A-21 (5' to 3' sequence = ACg gYT ACC TTg TTA CgA CTT).[18] The conditions for PCR included: 5 min. at 94°C; 30 cycles of 0.5 min. at 94°C, 0.5 min. at 55°C, and 0.5 min. at 72°C; 7 min at 72°C; and hold at 4°C. Each reaction tube contained: 1.25 U Takara Ex Taq polymerase,[9] 1x Takara Ex Taq reaction buffer, 200 µM of each deoxy ribonucleotide triphosphate (dNTP), 0.2 µM of each primer, and 500 ng of genomic DNA. PCR was conducted using a model 2400 thermal cycler.[8]

Agarose gel electrophoresis was used to check the quality of the PCR product. A 1% (wt./vol.) agarose gel was prepared in 1 x tris buffered EDTA (1 x TBE is 90mM tris borate and 2 mM ethylenediamine-tetraacetic acid [EDTA]) according to the manufacturer's instructions.[19] Electrophoresis was conducted for two hours using a setting of 100 V for the power supply. DNA fragments were visualized with a hand-held UV lamp after staining the agarose gel for ten minutes at room temperature with 50 mg/l of ethidium bromide.

The PCR products were cloned into component cells of *E. coli* using the TOPO TA cloning kit, version K2[10] according to the manufacturer's instructions. The blue/white screen with x-gal was used to detect the presence of insert in each plasmid, and the antibiotic ampicillin was used to screen for the presence of plasmids in colony-forming units of competent cells. Ten clones were selected for each team of students, and plasmid DNA was prepared using Perfectprep Plasmid Mini preps[11] according to the manufacturer's instructions. Purified plasmid DNA was subjected to endonuclease restriction analysis using *EcoR*I.[20] Digested plasmid DNA was electrophoresed on 2% (wt./vol.) agarose gels and visualized using ethidium bromide staining and a hand-held UV lamp as described above.

Two clones from each team were selected for commercial, automated dideoxy terminal sequencing by the DNA Core Facility at the University of Cincinnati. Sequencing primers included M13(-20) forward and M13 reverse[10] as well as S-*-Bact-0343-a-A-15 (5' TAC ggg Agg CAg CAg 3'), S-*-0519-a-S-18 (5'gTA TTA CCg Cgg CTg CTg 3'), S-*-Bact-0907-a-A-20 (5' AAA CTC AAA TgA ATT gAC gg 3'), and S-*-Bact-a-S-16 (5' Agg gTT gCg CTC gTT g 3').[18]

Step 4 • An initial phylogenetic analysis was conducted, and the results were used to design oligonucleotide hybridization probes for fluorescence in situ hybridization (FISH). Assembled sequences were compared to the Ribosome Database Project (RDP) (available at rdp.cme.msu.edu) using Chimera Check and Probe Match. Preliminary phylogenetic affiliation was confirmed using a BLAST (Basic Local Alignment Search Tool) search of GenBank (available at www.ncbi.nlm.nih.gov, follow the links to BLAST). The fluorescently labeled oligonucleotide probes were ordered from a commercial vendor.

Step 5 • Each team conducted fluorescence in situ hybridization (FISH) analysis of their original samples. Aliquots of the original sample were chemically "fixed" for one hour at room temperature with 4% (wt./vol.) paraformaldehyde prepared in 1 x phosphate buffered saline (1 x PBS is 130 mM NaCl and 10 mM sodium phosphate buffer). The samples were subsequently stored at -20°C in a 50% (vol./vol.) mixture of ethanol and 1 x PBS. The fixed samples were applied in a sample well on a Heavy Teflon Coated microscope slide[21] and air-dried. FISH was performed as previously described.[22] Briefly, each microscope slide was dehydrated in an increasing ethanol series (50, 80, and 95% [vol./vol.] ethanol, one minute each), each sample well was covered with 9 µl of

hybridization buffer (20% [vol./vol.] formamide, 0.9 M NaCl, 100 mM Tris HCl [pH 7.0], 0.1% SDS), and fluorescently labeled oligonucleotide probe, 1 µl (50 ng), was added to each sample well. Hybridizations were conducted in a moisture chamber for two hours, in the dark, at 46°C. The slides were washed for 30 minutes at 48°C with 50 ml of prewarmed wash solution (215 mM NaCl, 20 mM Tris HCl [pH 7.0], 0.1% SDS, and 5 mM EDTA). Fixed, hybridized cells were mounted with Cargille immersion oil[23] and a cover slip. Probe-conferred fluorescence was visualized with a model E600 upright epifluorescence microscope,[24] and digital images were captured using a Spot-2 charge coupled device (CCD) camera.[25] The results of the FISH analysis included determining the abundance and spatial organization of phylogentically defined microbial populations identified by unique oligonucleotide hybridization probes.

The students learned the procedures for the laboratory exercises through a video series produced specifically for this course. They were given a laboratory manual at the start of the class, and videos of the laboratory exercises were distributed biweekly in VHS format. The manual outlined all of the procedures for the laboratory and provided step-by-step instructions to complete each exercise. The videos gave the students an opportunity to view the instructor completing all of the steps of each exercise. The laboratory exercises were completed independently by the three-student teams according to a schedule arranged at the start of the class. Approximately the first fifteen minutes of the weekly lectures were dedicated to reviewing the progress of each team toward meeting the schedule for completion of the laboratory exercises.

TOPICS FOR THE LECTURES

Each week, approximately two hours were spent in a lecture discussion format with the entire class. The nine topics that were covered in the pilot course included:

- Overview of methods including the value of different methods and an answer to the question, "Why do Environmental Engineers need to learn molecular biology?"
- Measuring microbial community structure
- Measuring microbial community function
- Quantitative molecular biology for Environmental Engineering versus qualitative molecular biology for Environmental Science
- Troubleshooting the laboratory exercises to improve the course for the subsequent year
- What is this "phylogeny stuff" anyway?
- Historical development of molecular tools in Environmental Science and Engineering
- Success stories for molecular tools in Environmental Science and Engineering
- Principles of microscopic examination

Sex	N=13	Age	N=13	Number of Previous Biology Courses	N=13
Male	5	<23	0	<2	2
Female	8	23-26	4	<5	4
		27-30	5	<10	1
		30+	4	<15	1
				15+	4
Current Degree	**N=13**	**Current Degree Field**	**N=13**	**Hours per week on course**	**N=13**
B.A.	1	Env. Eng.	5	<4	2
B.S.	4	Env. Sci.	1	<6	7
M.S.	7	Engineering	2	<8	1
Ph.D.	1	Other	5	<10	2
Highest Degree	**N=13**	**Highest Degree Field**	**N=13**	<12	
				<15	1
M.S.	4	Env. Env.	8		
Ph.D.	9	Env. Sci.	5		

Figure 2. *Demographic of students enrolled in the pilot course as determined by an anonymous, in-class survey.*

STUDENT FEEDBACK

Figure 2 summarizes the results of students' responses to a demographic survey. Thirteen of the fifteen students enrolled in the course responded to the survey. The class was divided almost equally between male and female students with a median age of 27-30 years old. Five of the students had received significant formal training in biology, previously participating in more than ten biology courses. The majority of the students had already completed their MS degree (eight out of thirteen), but more than 50% of the students had received their degree outside of environmental engineering or environmental science. Most students spent less than six hours per week on the course, but some students spent significantly more time. Overall, the students enrolled in the pilot test of "Molecular Methods in Environmental Engineering" could be categorized as mature students (*i.e.*, in their late twenties working toward their doctoral degrees). Furthermore, the class contained a significant number of students with extensive previous experience in biology. Thus, the students enrolled in the pilot course were well prepared in maturity and previous biology experience to actively participate in this novel course. As the course continues to be offered, I plan to track the success of the course in relationship to the demographics of the enrolled students.

In addition to collecting demographic information, at the end of the class the students were asked to respond to three open-ended questions. In response to the question, "In your opinion, were the objectives of the course met?" students responded:

- *The course met some of the objectives, but some students*

are not convinced why we use molecular biology to identify microorganisms in systems that have been proved or have been operating successfully.

- *Yes. I am equipped with knowledge about this approach, and I can interpret research results and publications from this developing field.*

In response to the question, "What was the best aspect of this course?" students responded:

- *Most of the procedures are basic/universal operations in molecular biology which means that we understand how to study biology and biotechnology at the molecular level.*
- *Experimental work—because it is through applications that a student gets a tight grip on ideas and concepts. In addition, the challenging experiments and the value of the final result make the work more interesting.*
- *The lectures were interesting and informative. I learned a great deal, and my ideas about environmental engineering and science have been positively affected by the knowledge I have gained.*
- *Your perspective. We will never see "cutting edge" developments in a book.*
- *The whole structure of the course is similar to a research project.*
- *The best aspect was carrying the concepts from the classroom to the lab in a manner relevant to our field. Also, having a class that is new gives a fresh perspective into the future of environmental engineering.*

In response to the question, "What part of the course would you suggest improving?" students responded:

- *More theoretical basis, especially for the background of molecular biology methods.*

From their responses to the open-ended questions, it is apparent that the students felt the pilot course was a success. It is interesting to note that the students appreciated that the pilot course represented an effort to integrate research into the classroom. One of the greatest difficulties for developing a role for molecular biology in an engineering curriculum is discovering a mechanism for moving these "state-of-the-art" research skills into a classroom setting. In the future, we plan to expand the enrollment for "Molecular Methods in Environmental Engineering" to include undergraduate environmental engineering students as well as graduate and undergraduate students from related disciplines, including chemical engineering and biomedical engineering.

CONCLUSIONS

To address the growing national need for integrating genomics and molecular biology into the engineering curriculum, the author developed and pilot tested a new course, "Molecular Methods in Environmental Engineering." Fifteen graduate students were successfully introduced to molecular biology through lectures and hands-on laboratory exercises following the "full-cycle 16S rRNA approach." Although the pilot course can be considered a success, future offerings of this course must be modified to reduce the difficulty of comprehending molecular biology by inexperienced engineering students. One of the most daunting challenges for this type of "state-of-the-art" course is providing a supportive, yet independent learning environment. For highly motivated graduate students, the author demonstrated that the format for this course is successful. To offer this course to undergraduate students or poorly prepared graduate students represents a future challenge. In upcoming course offerings, the author plans to open enrollment for "Molecular Methods in Environmental Engineering" to undergraduate students in environmental engineering as well as students in chemical engineering and biomedical engineering. As genomics and molecular biology become as common to an engineering curriculum as chemistry and physics, engineering faculty need to take the lead in developing courses that introduce these topics from an engineering perspective with a focus upon quantitative approaches and the application of science to find cost-effective solutions to society's problems.

ACKNOWLEDGMENTS

This laboratory course would not have been possible without the commitment of significant resources from the Department of Civil and Environmental Engineering of the University of Cincinnati. For the success of the pilot test, the author is grateful to the Department.

REFERENCES

1. Amann, R., W. Ludwig, and K.H. Schleifer, "Phylogentic Identification and In Situ Detection of Individual Microbial Cells without Cultivation," *Microbiol. Rev.*, **(59)**, p. 143, (1995)
2. Rittman, B. "Editorial: Molecular Understanding," *Water Environ. Res.*, **(70)**, p. 1107, (1998)
3. Stensel, H.D., 2001, "Editorial: Probing the Black Boxes, *Water Environ Res.*, **(73)**, p. 259, (2001)
4. Woese, C.R., "Bacterial Evolution," *Microbiol. Rev.*, **(51)**, p. 221, (1987)
5. Woese, C.R., "There Must be a Prokaryote Somewhere: Microbiology's Search for Itself," *Microbiol. Rev.*, **(58)**, p. 1, (1994)
6. Hugenholtz, P., B.M. Goebel, and N.R. Pace, "Impact of Culture-Independent Studies on the Emerging Phylogenetic View of Bacterial Diversity," *J. Bact.*, **(180)**, p. 4765, (1998)
7. Catalog # 12800-100, MoBio, Solano Beach, CA
8. Applied Biosystems, Foster City, CA
9. PanVera Corp., Madison, WI
10. Invitrogen Corp., Carlsbad, CA
11. Eppendorf Scientific, Westbury, NY
12. Model Ultima II, Revco, Inc., Asheville, NC
13. Biospec Products, Bartlesville, OK
14. Catalog # CSSU1214 and EC105, E-C Apparatus Corp., Holbrook, NY
15. Spectronic Unicam, Rochester, NY
16. Model C24, New Brunswick Scientific, Edison, NJ
17. Model E600, Nikon, Inc. Melville, NY

18. de los Reyes, M.F., F.L. de los Reyes, M. Hernandez, and L. Raskin, "Quantification of Gordona amarae Strains in Foaming Activated Sludge and Anaerobic Digester Systems with Oligonucleotide Hybridization Probes," *Appl. Environ. Microbiol.*, **(64)**, p. 2503, (1998)
19. E-C Apparatus Corp., Holbrook, NY
20. Promega, Inc., Madison, WI
21. Cel-Line Associates, New Field, NJ
22. Oerther, D.B., J. Pernthaler, A. Schramm, R. Amann, and L. Raskin, "Monitoring Precursor 16S rRNA of Acinetobacter spp. in Activated Sludge Wastewater Treatment Systems," *Appl. Environ. Microbiol.*, **(66)**, p. 2154 (2000)
23. Type FF, Cedar Grove, NJ
24. Nikon Instruments, Inc., Melville, NY
25. Diagnostic Instruments, Inc. Sterling Heights, MI ❐

ChE letter to the editor

To The Editor:

This letter is motivated by the paper "An Undergraduate Course in Applied Probability and Statistics" that appeared in the Spring 2002 issue of *Chemical Engineering Education*.[1] Probability and statistics are difficult subjects to teach to engineering students, and Professor Fahidy is to be congratulated on his efforts in this area.

In this letter we would like to refer to the discussion and examples related to regression analysis. Professor Fahidy discusses in detail the use of numeric information (such as error variance, confidence intervals, correlation coefficient, etc.) for regression analysis, but does not mention graphic information (residual plots) and physical insight for regression analysis. Using the examples presented by Professor Fahidy,[1] we would like to demonstrate the importance of including graphical information and physical arguments in the regression analysis.

Let us refer first to Example 4 in the paper. In this example, the integral method of rate data analysis is used for a (supposedly) first-order reaction. Nonlinear regression can be used for finding the reaction rate coefficient (k) using concentration (Y) versus time (t) data, on the regression model Y = exp(-kt). Alternatively, this equation can be linearized to yield lnY=-kt, where linear regression can be applied. The results of the linear and nonlinear regression that were obtained using POLYMATH 5.1 are shown in the first two columns of Table 1. Note that these results are different from what is presented in [1], but they are correct and were confirmed by the author of the original article.[2] Looking at the numerical information presented in Table 1 (parameter values, confidence intervals, correlation coefficients, and variances) leads to the conclusion that there is no significant difference between linear and nonlinear regression for determining k (the variances are almost the same, contrary to what is argued in [1]). The same information may also lead to the conclusion that the model fits the data reasonably well. This conclusion, however, is contradicted by the residual plot shown in Figure 1. The residuals are not randomly distributed around a zero value. This may indicate either lack of fit of the model, or that the underlying assumption of a random error distribution for the dependent variables is incorrect.

TABLE 1
Regression Results for Example 4 in Reference 1

Reaction Order	*1st Order*	*1st Order*	*0th Order*	*2nd Order*
Model	logY=-k*t	Y=exp(-k*t)	$Y=Y_0+k*t$	$1/Y=1/Y_0+k*t$
k (value)	0.0039888	0.0038126	-0.0042162	0.0059893
95% Conf. Interval	±0.0011009	±0.0010816	0.0015209	±0.0059893
Y_0 (or $1/Y_0$, value)	-	-	1.0329275	0.9365288
95% Conf. interval	-	-	±0.586582	±0.1012594
R^2	0.7620164	0.7770319	00.8362884	0.7757433
Variance (based on Y)	0.0023055	0.002271	0.0018759	0.0021994

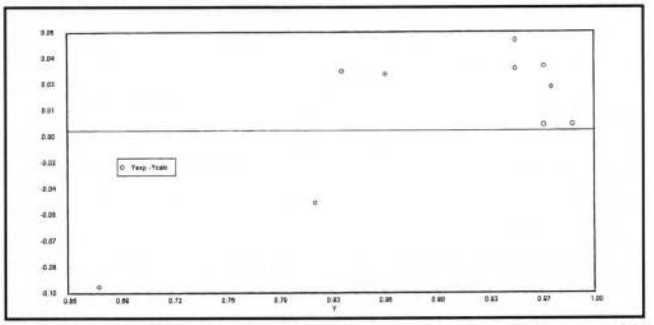

Figure 1. *Residual plot for Example 4 in Fahidy paper.*[1]

Physical insight can suggest alternative regression models, but more information regarding the reaction involved is needed. Since no such information is available, we will assume a homogeneous reaction, just for the sake of the demonstration. Assuming 0^{th} order reaction or 2^{nd} order reaction yields the models shown in the third and fourth columns of Table 1, respectively. The numeric information presented in the Table points on the 0^{th} order reaction as the most appropriate one (smallest variance value—note that in order to be on a unique scale, all the variance calculations must be based on Y). The residual plot for the 0^{th} order reaction is not significantly different, however, from that shown in Figure 1; thus, this model is not supported by the residual plot either.

The conclusion from proper analysis of this example is that the data available are insufficient (in quality, quantity, or both) to determine in any certainty the order of the reaction it represents. To obtain a more definite result, additional measurements must be made.

In Example 5, a linear model Y=a+bx is fitted to data of mean fuel consumption rate (Y) versus vehicle mass (x). The numerical results that were obtained for this example, using POLYMATH, are: parameter values (including 95% confidence intervals) a=-0.8695975±2.0733031;

Continued on page 277